The effect of Binding Immunoglobulin Protein on the induction of regulatory B-cells during Mycobacterium *tuberculosis* (*M.tb*) infection

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Declaration

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Abstract

Regulatory and killer function in B-cells have been described during tuberculosis (TB) disease, and these were shown to regulate inflammation through several mechanisms including both cytokine secretion (IL-10, IL-35, TGF-β, sFas-L and Granzyme-B) and cell surface expression of Fas-L, PD-L1 and FoxP3.

During TB treatment, binding immunoglobulin protein (BiP) secretion was assessed through Enzyme Linked ImmunoSorbent (ELISA) assay. This included plasma samples from healthy controls (n=32), TB diagnosis (n=29), week-1 follow up (n=8), month-2 follow up (n=7), and month-6 follow up (n=19) with additional collection from 20 participants (month-6 total (n=39)). Increased detection of BiP in plasma was observed between TB diagnosis and week-1 follow up suggesting a metabolism shift due to cell stress.

The effect of extracellular BiP on immune cell responses were determined by stimulating Peripheral Blood Mononuclear Cells (PBMCs) isolated from healthy controls (n=12) and Mycobacterium *tuberculosis* (*M.tb*) exposed participants (LTBI) (n=8) with human recombinant BiP at 20 µg/ml. This effect was compared to other antigenic material including Toll-like Receptor-9 agonist (TLR-9a), *M.tb* H37Rv, Isoniazid (INH), BiP+TLR-9a, pooled Bronchoalveolar Lavage (BAL) fluid at TB diagnosis (TBdx) and month 6 TB treatment (M6) with unstimulated PBMCs as a baseline control. From this, the cytokine profile was established which indicated an elevated level of sFas-L and IL-13 by BiP stimulation. Further, increased frequency of $CD19^{+}CD5^{+}$ B-cells co-expressing Fas-L and IL-5Rα was greatly induced by BiP in both healthy controls and LTBI participants. Kinase activity was assessed by Luminex multiplex assay between unexposed group, *M.tb* exposed and stimulation conditions. Taken together, this study shows BiP potential to aid in better *M.tb* control by upregulating a B-cell population with immune regulatory function through expression of Fas-L. This highlights the potential use of BiP in host directed therapies (HDT) during TB disease to aid in better infection response.

Opsomming

Regulerings- en vernietigingsaksie in B-selle is tydens tuberkulose (TB) siekte beskryf, en daar is getoon dat ditinflammasie deur verskeie meganismes reguleer, insluitende sitokiensekresie (IL-10, IL-35, TGF-β, sFas-L en Granzyme -B) en seloppervlakte uitdrukking van Fas-L, PD-L1 en FoxP3. Tydens TB behandeling, is bindende immunoglobulien proteïen (BiP) sekresie geassesseer deur ‘n Enszyme Linked ImmunoSorbent Assay (ELISA) toets. Plasma monsters van gesonde pasiënte (n = 32), TB pasiënte tydens diagnose (n = 29), week 1 opvolg (n = 8), maand 2 opvolg (n = 7) en maand-6 opvolg n = 19) met die bykomende versameling van 20 deelnemers (maand-6 totaal (n = 39)) is ingesluit. Verhoogde BiP vlakke in plasma is waargeneem tussen TB diagnose en week 1 opvolg wat 'n metabolisme verskuiwing weens selspanning aandui.

Die effek van ekstrasellulêre BiP op immuun sel response is bepaal deur die stimulering van perifere bloed mononukleêre selle (PBMCs) wat geïsoleer is van gesonde kontroles (n = 12) en Mycobacterium *tuberculosis* (*M.tb*) blootgestelde deelnemers (LTBI) (n = 8) met menslike rekombinante BiP van 20 μg/ml. Hierdie effek is in vergelyking met ander antigeenmateriaal, insluitend Toll-like Receptor-9-agonist (TLR-9a), *M.tb* H37Rv, Isoniazid (INH), BiP + TLR-9a, saamgevoegde Bronchoalveolêre spoeling (BAL) vloeistof by TB diagnose (TBdx) en maand 6 TB behandeling (M6) met ongestimuleerde PBMCs as 'n basislynbeheer. Hieruit is die sitokienprofiel vasgestel wat 'n verhoogde vlak van sFas-L en IL-13 deur BiP-stimulasie aangedui het. Verder is gevind dat verhoogde frekwensie van $CD19^{+}CD5^{+}$ B-selle wat Fas-L en IL-5Rα terselfdetyd uitdrukveroorsaak word deur BiP in beide gesonde pasiënteen LTBI-deelnemers. Kinase-aktiwiteit is geassesseer deur Luminex-multipleks assessering tussen die nie-blootgestelde groep, *M.tb* blootgestel en stimulasie toestande. Hierdie studie het dus getoon dat BiP potensiaal het om *M.tb* beheer te verbeter deur 'n B-sel bevolking met immuunregulerende funksie te reguleer deur die uitdrukking van Fas-L. Hierdie resultatedui op diedie potensiële gebruik van BiP in gasheergerigte terapieë (HDT) tydens TB-siekte om beter infeksierespons te bevorder.

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List of abbreviations

ATF6	Activating Transcription Factor 6
APC	Antigen Presenting Cell
BiP	Binding Immunoglobulin Protein
BCG	Bacillus Calmette-Guerin
BCR	B-cell Receptor
B-reg	Regulatory B-cell
CD	Cluster of Differentiation
CHOP	C/EBP homologous protein
CIA	Collagen Induced Arthritis
CTRL	Control group
ER	Endoplasmic Reticulum
EAE	Experimental Autoimmune Encephalomyelitis
GRP78	Glucose Regulated Protein 78
H37rv	Mycobacterium tuberculosis strain
HDT	Host Directed Therapy
HLA-DR	Human Leukocyte Antigen DR
IL	Interleukin
INH	Isoniazid
IRE-1	Inositol Requiring Enzyme 1

LTBI	Latent tuberculosis infection
M2	Month-2 anti-TB treatment
M6	Month-6 anti-TB treatment
MFI	Mean Florescent Intensity
MHC	Major Histocompatibility Complex
M.*tb*	Mycobacterium tuberculosis
OD	Optical Density
pAPC	professional Antigen Presenting Cell
PBMC	Peripheral Blood Mononuclear Cell
PD-L	Programmed Death Ligand
PERK	Protein Kinase-like ER kinase
p-value	Probability value
QFN	Quantiferon
RA	Rheumatoid Arthritis
TB	Tuberculosis
TBdx	Tuberculosis disease diagnosis
TGF-β	Transforming Growth factor beta
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
UPR	Unfolded Protein Response

W1	Week-1 anti-TB treatment
WHO	World Health Organization

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Chapter 1

Introduction

Background

Mycobacterium tuberculosis (*M.tb*), a causative agent of tuberculosis (TB) disease which has been reported by The World Health Organisation (WHO) to be responsible for 1.3 million deaths within HIV negative population in 2017 while leaving 10 million people latently infected (WHO, 2018). This pathogen can easily be transmitted from one individual to the next through aerosol particles where it primarily infect the lungs (Fennelly et al., 2004; Smith, 2003). It can migrate to other body organs such as eyes, spine, lymph nodes and meninges (Jawahar et al., 2005; Panicker et al., 2016). During infection, *M.tb* uses aerosol particles as a vector to enter through airways into the lung alveolar space where it is taken up by phagocytic cells such as macrophages (Delogu et al., 2013; Woo et al., 2018). This bacterium is classified as an intracellular pathogen and has evolved mechanisms to escape its host immune system by supressing major histocompatibility complex (MHC) presentation of infected cells and also prevents membrane repair in infected cells (Divangahi et al., 2009; Noss et al., 2000). It is also suggested that virulent *M.tb* avoids the formation of phago-lysosome by preventing fusion between phagosome vesicles and lysosome thus allowing it to persist longer (Flynn and Chan, 2003; Russell, 2001). Infected phagocytes secretes proinflammatory cytokines and chemokines that further recruit other immune cells resulting in the formation of an organized structure called granuloma (Domingo-Gonzalez et al., 2016). In this state, *M.tb* can survive for years within its host without causing any noticeable clinical characteristics. This phase is referred to as latent or quiescent stage and is characterized by proper containment of the pathogen (Flynn and Chan, 2003, 2001). However, within these structures, there arecontinuous interactions between uninfected immune cells

and infected cells (Silva et al., 2018). Alterations in this effective communication lead to necrotic death of infected cells thus releasing the bacteria to infect more surrounding cells (Michelet et al., 2018). Continuous necrotic cell death of infected cells eventually leads to granuloma dissemination resulting in active TB disease.

Secreted cytokines facilitate activation and recruitment of other immune cells to the site of infection (Davis and Ramakrishnan, 2009). Their secretion varies with the type of inflammation and antigenic material. Similarly, *in vitro* stimulation of isolated cells with different antigens result in differential cytokine profiles both within the pro- and anti-inflammatory responses (du Plessis et al., 2016a). Among the major pro-inflammatory cytokines secreted during *M.tb* infection is interferon gamma (IFN-γ), TNF-α and IL-1β which serves to activate macrophages and induce MHC II expression on lymphocytes. However, it has become evident that secretion of anti-inflammatory cytokines (such as IL-10, IL-35 and TGF-β) are also upregulated during inflammation (Ray et al., 2012; Shen et al., 2014). Dysregulation in immune response may lead to either elevated levels of pro-inflammatory cytokines and aggressive immune responses or extremely suppressed immune responses by anti-inflammatory cytokines. In the context of TB disease, research platforms focused on effector or pro-inflammatory functions; thus, suggesting the need to further explore regulatory functions mediated by immune cells during this disease. Successful clearance of *M.tb* pathogen from the host requires a precise balance between pro- and anti-inflammatory responses, this is thought to be possible through host directed therapies (HDT). Induction of autophagy and apoptosis has been observed as one of the promising interventions in HDT during *M.tb* infection (Rashid et al., 2015).

This infection is characterized by a delayed adaptive immune response which further allows the survival of the pathogen at the site of infection (Cooper, 2009; Wolf et al., 2008). During this time, immune responses are mediated by innate cells. Interestingly, B-cells have been shown to mediate both arms of immune responses; with antigen presentation during innate function and antibody and

cytokine secretion during adaptive responses (du Plessis et al., 2016b) which are mainly mediated by T-cells of both $CD4^+$ T-helper and $CD8^+$ cytotoxic T-cell subsets (Tan et al., 1997; Wolf et al., 1996). During *M.tb* infection, these T-cell subsets are activated through recognition of short and long *M.tb* peptides which are displayed on MHC I and II by APCs. Regulatory cells regulate effector cell functions; these arise from B- and T-lymphocytes; and has been implicated to participate in *M.tb* control through secretion of anti-inflammatory cytokines and induction of apoptosis in infected cells (Hirsch et al., 2016; Iwata et al., 2011; Oddo et al., 1998). A subpopulation of B-cells with regulatory function expressing death ligands (Fas-L and PD-L1) has been defined during both inflammation and healthy state (Khan et al., 2015; van Rensburg et al., 2017; van Rensburg and Loxton, 2018; Wang et al., 2017). However, factors inducing this phenotype in B-cells remain unknown and so does their role during chronic TB disease. Availability of binding immunoglobulin protein (BiP) in extracellular fluids has been linked with the induction of immune regulatory functions during rheumatoid arthritis and other autoimmune diseases (Bläss et al., 2001; Shields et al., 2015; Yoshida et al., 2011). However, this has never been evaluated during chronic inflammatory conditions such as TB disease.

Even though B-cell functions are crucial during inflammation, these cells were reported in reduced frequencies during TB disease (Joosten et al., 2016). B-cells are activated through engagement of their B-Cell Receptor (BCR) with soluble or membrane-bound antigens. Activated B-cells have increased expression of surface immunoglobulin CD38 (Damle et al., 2007). This activation further leads to differentiation and expansion within B-cell population which is indicated by increased expression of surface IL-5Rα (Katoh et al., 1993). During *M.tb* inflammatory responses, B-cells mediate several functions which involve various B-cell differentiation such as plasma cells, killer and regulatory B-cells. Regulatory characteristics in B-cells is associated with increased IL-10 secretion which is inducible at any stage during B-cell development (Iwata et al., 2011). However, characterization of their exact phenotype and function during TB disease is still a growing field. Regulatory B-cells have been previously defined at varying frequencies within $CD5^+$, $CD5^+CD1d^+$

and $CD24^{+}CD27^{+}CD38^{+}$ B-cell populations (van Rensburg et al., 2017; van Rensburg and Loxton, 2018; Yanaba et al., 2008; Zhang et al., 2012). These cells mediate two main functions which involve maintaining invariant Natural killer cells (iNK) homeostasis and immunosuppression. They suppress immune responses by affecting T-cell differentiation, mainly T-helper type 1 (Th1) and 17 (Th17) cells through suppression of pro-inflammatory cytokines produced by antigen presenting cells (APC) (Zhang et al., 2012). Apart from secreting IL-10 as a major anti-inflammatory cytokine, B-regs can also suppress immune responses through IL-35 and TGF-β cytokines which regulate immune responses by stimulating apoptotic pathways in $CD4^{+}$ and $CD8^{+}$ T-cell (Rosser *et al*., 2015). Additionally, expression of Fas-L and PD-L1 on these cells enhance their immune regulatory function through induction of apoptotic pathways in metabolically exhausted cells flagged with receptors for these ligands. Regulatory traits in B-cells can be induced by various antigens including Toll-Like Receptor-9 agonist (TLR-9a), bacterial Lipopolysaccharide (LPS), and Bacille-Calmette Guerin (BCG). However, mechanisms involved in this are largely unknown and needs to be further studied (du Plessis et al., 2016a; van Rensburg and Loxton, 2018). A study by Tang et al., (2016) using mouse models showed that extracellular BiP can upregulate expression of Fas-L, IL-10 and PD-L1 by isolated B-cells *in vitro*.

Expression of these cytokines and surface markers is modulated by metabolic pathways and transcription factors within the cell. These pathways are modulated with respect to the extracellular environment and calcium homeostasis as main factors which are highly important for cell activation, survival and differentiation. During B-cell activation through the binding of B-cell Receptor (BCR) to antigenic material, phosphoinositol-3-kinase (PI3K) becomes phosphorylated and activated which further mediate the Akt/mTOR pathway (Fruman and Limon, 2012). It has been shown that B-cell proliferation and functionality highly depend on this pathway and this has been reported to be greatly enhanced by a Th2-cell cytokine IL-4 (Fruman and Limon, 2012; Okkenhaug and Vanhaesebroeck, 2003). Akt/mTOR pathway plays a central role in calcium homeostasis, activation of protein kinase

C and translocation of necrosis factor kappa beta (NFκβ) transcription factors, all of which are vitally important for cellular function. Formation of auto-phagosomes has been shown to depend on mTOR modulation among others, which include inositol signalling pathway, calcium/ calpain signalling and cyclic adenoside monophosphate (cMAP) (Jung et al., 2010). mTOR Activation inhibits autophagy formation whereas its blockage enhances the formation of auto-phagosomes (Ravikumar et al., 2004). These conditions are also known to upregulate ER stress sensors which are involved in unfolded protein response (UPR). The ER stress response pathway, UPR, is regarded as a survival mechanism that cells use during high demand of immunoglobulin. This pathway promotes cell survival through various mechanisms which include the destruction of synthesized proteins, transcription and translation termination (Gass et al., 2002). However, it has also been shown that prolonged ER stress responses promote autophagy and activation of apoptotic pathways within the cell (Rashid et al., 2015). This response is mainly mediated by BiP, an ER chaperone protein that can act as an autoantigen when released to extracellular fluids (Bläss et al., 2001) and cause functional changes on immune cells during chronic inflammation such as arthritis rheumatoid, cancer and allograft.

Research gap and study rationale

Challenges associated with *M.tb* infection control are reported worldwide. Infected immune cells are known to undergo necrotic cell death thus promoting further spread of the pathogen within its host; this highlights the need to establish new strategies directed at host immune responses that can lead to better infection control and shortened treatment time. This may be achieved through manipulation of the host immune system which may involve suppression of adaptive immune responses and induction of apoptotic pathways in infected cells. A B-cell subset expressing an anti-inflammatory cytokine (IL-10) that suppresses pro-inflammatory responses mediated by T-cells during TB infection was demonstrated to be inducible (du Plessis et al., 2016). Furthermore, expression of apoptosis-inducing ligand (Fas-L) and receptor (PD-1) was defined by van Rensburg et al., (2017) on B-cells during TB treatment and subsequently shown to be inducible by BCG during latent TB infection (van Rensburg

and Loxton, 2018). However, these B-cell characteristics were shown to be depleted during active TB disease characterized by prolonged inflammatory responses to *M.tb*. This highlighted the need to further explore development and functions mediated by these B-cell subpopulations during *M.tb* infection. Currently, there is limited data addressing induction and functional responses mediated by these cells during TB disease. Therefore, this study will increase our understanding of B-regs frequencies in the Peripheral Blood Mononuclear Cells (PBMC) from both healthy and latently infected participants and how these cells respond to extracellular BiP during active TB disease.

Hypothesis

BiP can induce anti-inflammatory response with increased expression of killer (regulatory) phenotype on B-cells during *M.tb* exposure.

Aim

To evaluate the effect of binding immunoglobulin protein (BiP) on induction of anti-inflammatory responses, killer (regulatory) B-cells and modulation of Akt/ mTOR during *M.tb* exposure.

Objectives

Overall objective

To characterize the effect of BiP on the peripheral blood mononuclear cells during exposure to *M.tb*.

Specific Objectives

Our aim was achieved through the following:

- Assess extracellular BiP secretion in plasma samples over the course of anti-TB treatment.
- Determine cytokine secretion from unstimulated and antigen (including BiP) stimulated cells using multiplex assays.
- Determine phenotypic changes in B-cells and T-cells by flow cytometry following antigenic stimulation with BiP.
- Assess intracellular pathway activation through targeting Akt/mTOR signalling.

Study groups description

Completion of this study involved participant groups at different TB stages regardless of human immunodeficiency virus (HIV) status; all participants were above 18 years and gave informed consent:

- Healthy participant/ control (CTRL) group; with negative QuantiFERON status and no previous traces of M.*tb* infection.
- Latently infected group; recently diagnosed QuantiFERON positive participants.
- Active TB at diagnosis (TBdx); characterised by positive QuantiFERON status, positive sputum cultures and positive lung x-ray profiles.
- Anti-TB treatment group at week 1 (W1)
- Anti-TB treatment group at month 2 (M2)
- Anti-TB treatment group at month 6 (M6)

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Chapter 2

Effect of Binding Immunoglobulin Protein (BiP) on inducing regulatory B-cells during inflammation and disease

The work presented here has been formatted in the style of Biomarkers in Medicine journal to which it currently submitted and under review.

Effect of Binding Immunoglobulin Protein (BiP) on inducing regulatory B-cells with killer phenotype during inflammation and disease

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Summary

Immune responses result from different immune cells acting in a synergistic manner to successfully fight infections. Nonetheless, these require a high degree of regulation to prevent excessive production of inflammatory products leading to other disease forms. This was previously defined as mediated by T-cells; however, growing studies indicate the involvement of B-cells in this regard. These are specialized types of B-cells that express and secrete specific regulatory markers (FasL, PD-L1 and GITR) and soluble substances (Granzyme-B, sFasL, TGF-β, IL10 and IL35). These subsets are classified based on surface immunoglobulin expression and their frequencies range from 0.1-12% of the total B-cell population. Their functionality has been reported to resolve inflammation during autoimmune diseases (such as rheumatoid arthritis and collagen-induced arthritis) and cancer. Chronic infection such as TB affect their frequencies, however, they have been shown to be inducible in a murine experiment using binding immunoglobulin protein (BiP). Here we define regulatory B-cell immunity and effects posed by BiP on the development of these cells and other immune cells. Cell stress associated with Mycobacterium tuberculosis (*M.tb*) infection lead to accumulation of unfolded proteins which subsequently activate BiP and 3 signal transducers (activating transcription factor 6 (ATF6), protein kinase-like ER kinase (PERK) and inositol-requiring enzyme-1 (IRE-1)). However, BiP can be translocated from endoplasmic reticulum (ER) to the extracellular environment where binds to immune cells and lead to functional changes.

Immunity, B-cells and regulatory B-cell responses during inflammation (autoimmune/ infection)

Immunological studies have shown that successful clearance of any invading pathogen depends on the effective balance between immune cells and their secreted products such as cytokines, antibodies, and chemokines. Depending on the nature of infection, immune cell balance can be altered through biological processes such as necrosis, pyroptosis, programmed cell death and apoptosis (Brennan and Cookson, 2000). These cellular processes are triggered mostly by intracellular pathogens such as *M.tb* which has evolved mechanisms of suppressing immune responses by effector T-cells through the release of bacterial vesicles that expresses lipoarabinomannan (LAM) and other lipoglycans (Athman et al., 2017). These bacteria suppress immune activation through recruitment of mesenchymal stem cells (MSC) which secretes immunosuppressive cytokine and nitric oxide (NO) (Raghuvanshi et al., 2010). Even though Bacillus Calmette-Guerin (BCG) vaccination has been in use for years as it stimulates immune system and shortens specific antibody response against *M.tb* infection, which targets LAM embedded on their cell wall (de L. Costello et al., 1992), there is still a need for advances that will better eradicate or control the infection. These antibodies are secreted by a subpopulation of B-cells (plasma cells) furthermore they facilitate rapid cell mediated immunity through pathogen opsonization and binding of their Fc gamma receptors (FcγR) with professional antigen presenting cells (pAPC) which result in internalization of the pathogen (de Valliere et al., 2005). However, M.*tb* is known to reside and multiply within these APC leading to the formation of granuloma structures (Davis et al., 2002; Davis and Ramakrishnan, 2009). Dissemination of these granulomatous structures and progression to active tuberculosis has been shown to affect the frequency of immunological cells such as circulating peripheral B-cells (Joosten et al., 2016; Ma et al., 2014). Tuberculosis (TB) pathogen takes advantage of this imbalance in the immune system and multiplies further, thus infecting more and more cells. Immune system inadequacy or manipulation by *M.tb* highlighted the importance of exploring other functions played by immune cell subtypes as a means to better control infection. It has become evident through research studies that regulatory

functions in different immunological cells, including B-cells, plays more than just a role of suppressing aggressive immune responses during autoimmune and infectious diseases. These regulatory subsets play a major role in balancing the immune system and facilitate better elimination and control of pathogens and resolving inflammation (Bernard A et al., 2018; Guo et al., 2015; Holan et al., 2014; Ray et al., 2012). Immune suppression functions are mediated by a group of specialized regulatory cells in the innate (myeloid derived suppressor cells (MDSCs) and Natural Killer (NK) cells) (Cooper et al., 2001; Serafini et al., 2008) and adaptive arms, mainly of the T (regulatory T cells (T-regs) and B-lymphocytes (regulatory B-lymphocytes (B-regs)) (Guo et al., 2015; Mahic et al., 2006) which expresses differential surface receptors and secretes varying range of cytokine profiles. Development of regulatory B-cells and other B-cells subtypes with different immune function (Figure 2.1) is enhanced by various factors including activated/ stimulated cellular pathway, type of stimulant and extracellular concentration of micronutrients (Holan et al., 2014). In particular, regulatory function in B-cells was first described in Experimental Autoimmune Encephalomyelitis (EAE) (Wolf et al., 1996). It was initially thought that the primary function of these B-regs was to maintain the immune environment until regulatory T-cells are matured enough to take over the role. As depicted in Figure 2.1 and Figure 2.2, these cells exert their effect through secretion of soluble proteins (blocking specific intracellular pathway) and expression of surface ligand molecules such as Fas-L, FoxP3 and Programmed Death (PD) ligand (Guo et al., 2015; Khan et al., 2015) which enhance interaction with cells bearing receptors for those specific ligands and induce apoptosis or programmed death.

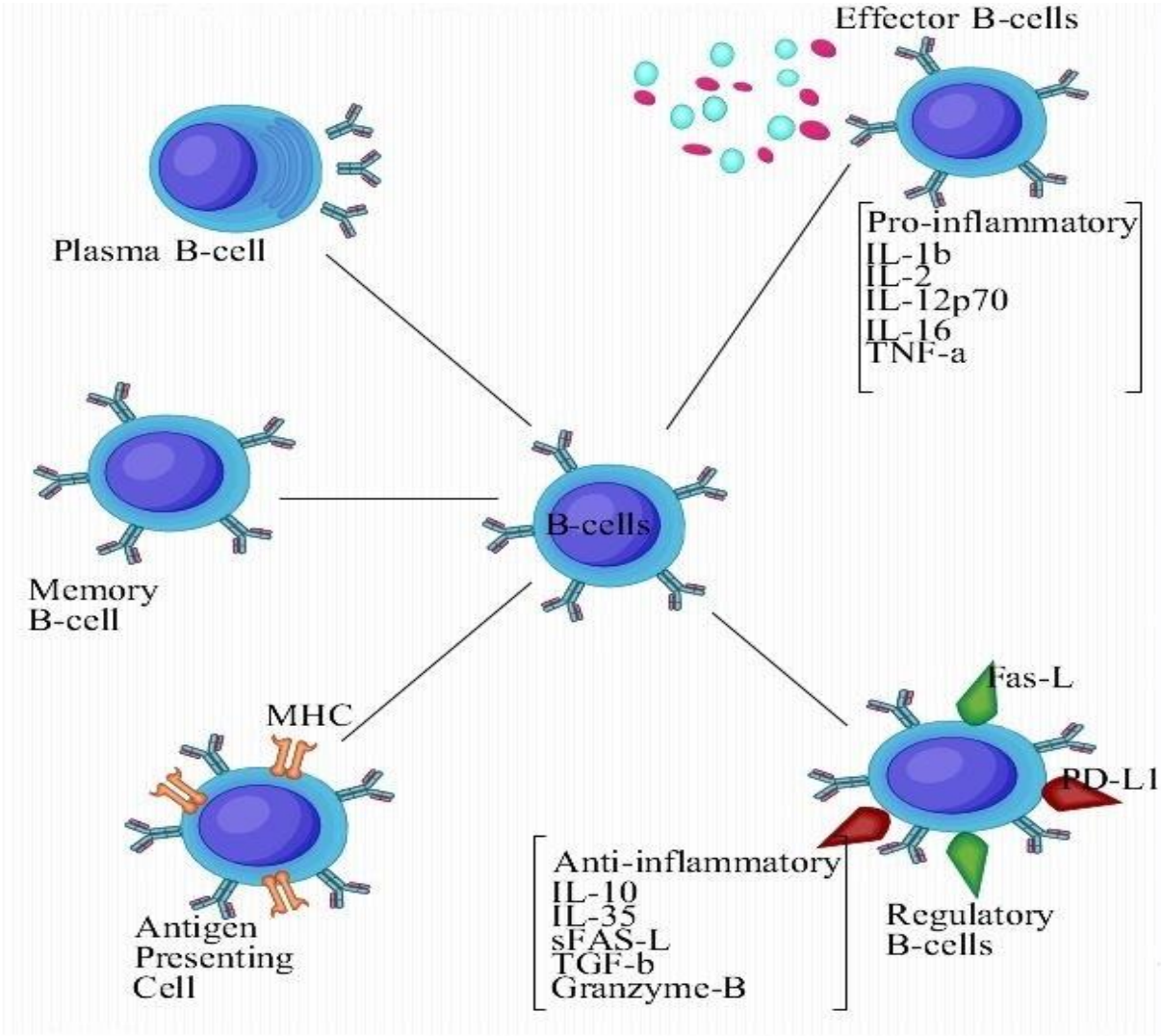

Figure 2.1: Different B-cell functional response to inflammation. Stimulation of any of the B-cell function depends on the nature of pathogenic material, whereas memory B-cells are long lasting immunological memory cells which bear specific receptors from the previous infection.

Regulatory B-cells have been implicated in many inflammatory studies including allograft tolerance, cancer, autoimmune diseases and infection (K. M. Lee et al., 2014; Li et al., 2016; Ma et al., 2014) where they have been shown to inhibit function and proliferation of T helper 1 and T helper 17 cells (Carter et al., 2011; Flores-Borja et al., 2013; Zhang et al., 2012). During autoimmune diseases, these cells increase tolerance of self-antigens and thus preventing the destruction of the body's own cells. Similarly, during infection inflammatory responses they limit the aggressiveness of the immune

system and prevent persisting immune responses after clearance of the pathogen. Even though B-regs have not been extensively studied during TB disease, current evidence suggests that B-cells with anti-inflammatory properties are present in smaller numbers in the peripheral stream and these decrease drastically during chronic infectious diseases (du Plessis et al., 2016a; van Rensburg et al., 2017a; Zhang et al., 2012). These cells are displayed at higher frequencies in healthy individuals and disappear over time during chronic immune responses thus leading to immune system imbalance (Joosten et al., 2016; Li et al., 2016; van Rensburg et al., 2017a). Regulatory B-cells are continuously reported to be dysfunctional during inflammation; furthermore, this is thought to be associated with exacerbation of disease state, especially in autoimmune diseases (Fillatreau et al., 2002).

Various antigens are known to drive the development of regulatory B-cells through upregulation of IL-10 secretion and these include BiP, BCG, lipopolysaccharide (LPS), infectious agents and Toll-like receptor 9 agonist (TLR-9a) (du Plessis et al., 2016b; LENERT et al., 2005; Tang et al., 2016). In particular, BiP has been shown to resolve inflammation in rheumatoid arthritis by upregulating anti-inflammatory cytokine production by immune cells (Corrigall et al., 2009; Yoshida et al., 2011). However, as depicted in Figure 2.2 biological pathways involved in the development of these cells by different antigens including BiP remains unknown.

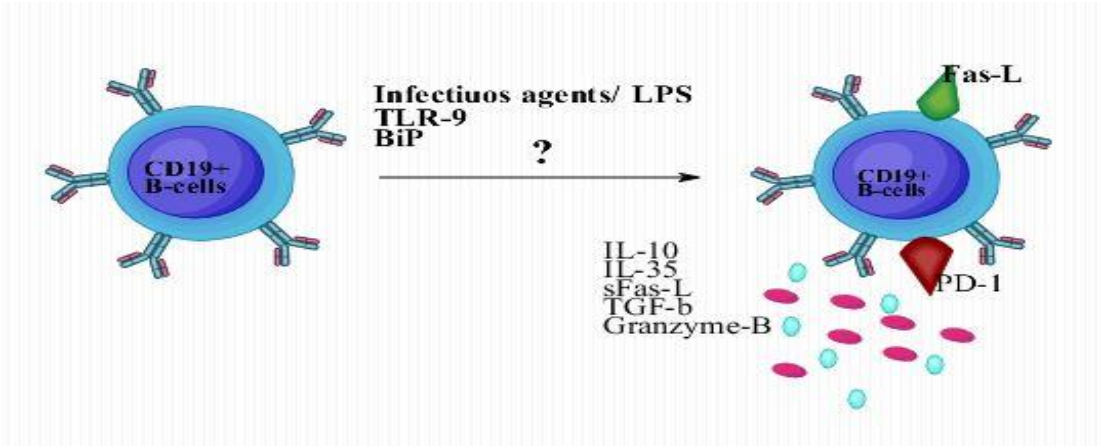

Figure 2.2: Biological pathways involved in the development of regulatory B-cells by various extracellular antigens have not yet been characterized and need further investigations.

Additionally, functional and phenotype characterization of these cells is still expanding in the context of different inflammatory conditions and new makers are still being identified which relates to extracellular environment composition during their development. It has been shown by recent studies that apart from IL-10 secretion, these cells are capable of secreting other soluble molecules including sFas-L, TGF-b, granzyme-B and IL-35 which plays crucial roles in suppressing inflammation (Guo et al., 2015; Tang et al., 2016; Zhang et al., 2016).

Phenotypic identified subsets of regulatory B-cells during inflammation.

Identification of regulatory B-cells is based on both extracellular surface immunoglobulin expression and cytokine profile, merely because these properties determine the mode of regulation by these cell types. As postulated in figure 2.3 there are four common categories of B-regs and these include IL-10 producing B-regs (commonly known as B*10* cells) which produce IL-10 as major cytokine (Yanaba et al., 2009), Transforming Growth Factor-β producing B-cells (B-*TGF-β*) and FoxP3 expressing B-cells (B-*FoxP3*) (Guo et al., 2015). Additionally, IL-35 is another cytokine secreted by regulatory B-cells, either alone or in conjunction with IL-10, to modulate immune responses during inflammation (Shen et al., 2014). Several phenotypes have been identified based on extracellular surface immunoglobulin expression within each B-regs category in various studies of both humans and murine. In humans, the majority of IL-10 expressing regulatory B-cells are represented by the expression of $CD19^{+}CD24^{hi}CD38^{hi}$, $CD1d^{hi}CD5^{+}$, $CD19^{+}TIM1^{+}$ on their surface (Ma et al., 2014; Matsushita et al., 2008; Yanaba et al., 2009; Zhang et al., 2012). Similarly, Shen et al., (2014) also described a sub-population of IL-35 producing regulatory B-cells differentiating from plasma cells expressing the phenotype $IgM^{+}CD138^{hi}TACI^{+}CRCX^{+}CD1d^{+}Tim1^{int}$ in murine (Shen et al., 2014). However, IL-21 has been shown to drive regulatory function in B-cells by upregulating expression of Granzyme-B in conjunction with IL-10. This subset has been phenotypically identified as $CD19^{+}CD38^{+}CD147^{+}CD1d^{+}IgM^{+}$ (Lindner et al., 2013). Contradicting results in relation to IL-10 were observed during a human immunodeficiency virus (HIV) study whereby $GraB^{+}$ B-regs did not

produce IL-10 (Kaltenmeier et al., 2015; Lindner et al., 2013). B-cell regulatory function linked with FasL and PD-L1 expression has been described in both inflammatory and healthy state in humans; these appear in varying frequencies in relation to inflammatory state and they were reported to be higher in healthy individuals than in infected individuals (Tang et al., 2016; van Rensburg et al., 2017a). $CD19^{+}FasL^{+}$ and $CD19^{+}PD\text{-}L1^{+}$ regulatory subsets secrete IL-10; although intracellular pathways are not fully exploited regarding IL-10 bio-synthesis by these B-cell subpopulations (van Rensburg et al., 2017a; van Rensburg and Loxton, 2018). However, there have been reports that significant amount of IL-10 by B-cells occurs through the involvement of Toll-Like Receptor (TLR) molecules rather than B-cell Receptor (BCR) or CD40 involvement (Liu et al., 2014). $CD1d^{hi}CD5^{+}$ B-cells were reported to be inducible for IL-10 secretion in significant amounts through TLR-9 using LPS and intracellularly mediated by MyD88/STAT3 pathway (Bénard et al., 2018; Liu et al., 2014; Yanaba et al., 2009). FoxP3 expressing B-cells in healthy human blood samples were first described in a study by Noh et al., (2010) within the $CD19^{+}CD5^{+}$ population. These cells appeared in higher frequencies within the $CD5^{+}$ group than in the $CD5^{-}$ group and they also showed to have increased apoptotic characteristics than other B-cell groups. A subsequent study by Guo et al., (2015) examined and described FoxP3 expressing $CD19^{+}$ B-cells to be of greater frequencies in healthy individuals than in patients with rheumatoid arthritis (RA). Although further studies still need to be done on $FoxP3^{+}$ B-cells, Guo et al., (2015) also compared their frequency with TGF-β $CD19^{+}$ B-cells and found no significant difference amongst the expression frequencies of these groups in both healthy and RA groups. Regulatory function and frequency of B-cells and other immune cells is affected by varying antigens present in their cellular environment.

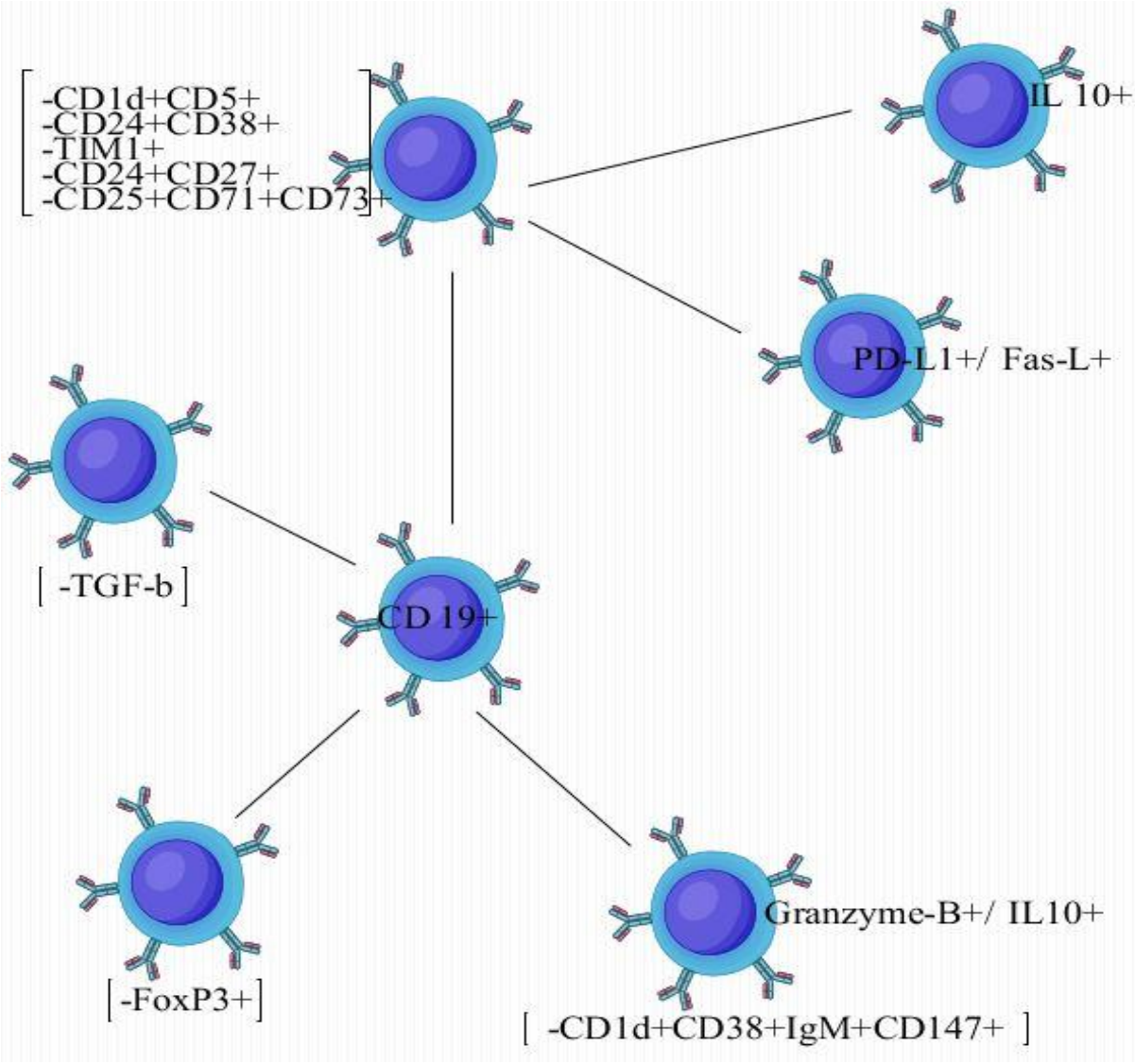

Figure 2.3: Circulating $CD19^{+}$ B-cells have the potential to develop to regulatory B-cells with different regulatory markers depending on their stage of development. Regulatory trait has been identified within B1 cells, Marginal Zone B-cells and Transitional-2 Marginal zone B-cells.

Binding Immunoglobulin Protein (BiP) affects immune cell function

BiP is a 78 kDa heat shock protein naturally occurring in the lumen of theER which aids in proper peptide folding (Yoo et al., 2012). Synthesis and expression of this protein have been reported to be upregulated during cellular stress which might be due to glucose starvation or in response to accumulation of unfolded proteins within the cell (Choi et al., 2010; Lee, 2005). Its activation/ synthesis was initially observed in a study by Munro and Pelham, (1986) where BiP was shown to be

expressed in high concentrations in antibody producing B-cells due to high levels of synthesized immunoglobulin inhabiting ER than in resting/ naive B-cells. Gass et al., (2002) exploited the Unfolded Protein Response (UPR) in B-lymphocytes and found it to be induced during B-cell transition to antibody secreting plasma cells thus suggesting a link between upregulation of this protein with immunoglobulin synthesis. Loss of BiP function in B-cells has been associated with an inability to secrete functional antibodies which may ultimately affect opsonization of invading pathogens (Hu et al., 2009). In particular, *M.tb* infection exerts stress onto immune cells through secretion of ESAT-6 which affects homeostasis of calcium ions and increase unfolded protein burden in cells due to metabolic shift. However, it has been implied that extended ER stress can lead to activation of apoptotic pathways in immune cells resulting in skewed immunological responses (Joosten et al., 2016; Li et al., 2006). As illustrated in Figure 2.4, ER stress activates and upregulate expression of BiP and other ER chaperones to mitigate the conditions posed onto cells (Choi et al., 2010). These chaperone proteins bind unfolded and partially folded proteins and direct them for proper folding in the ribosomes (Maddalo et al., 2012). They also act as co-activators of three ER membrane signal transducers; PERK, IRE1 and ATF6 (Figure 2.4), which in turn phosphorylates transcription factors and cytosol kinase involved in translation termination and cytokine synthesis. These mechanisms either promote cell apoptosis or cell survival.

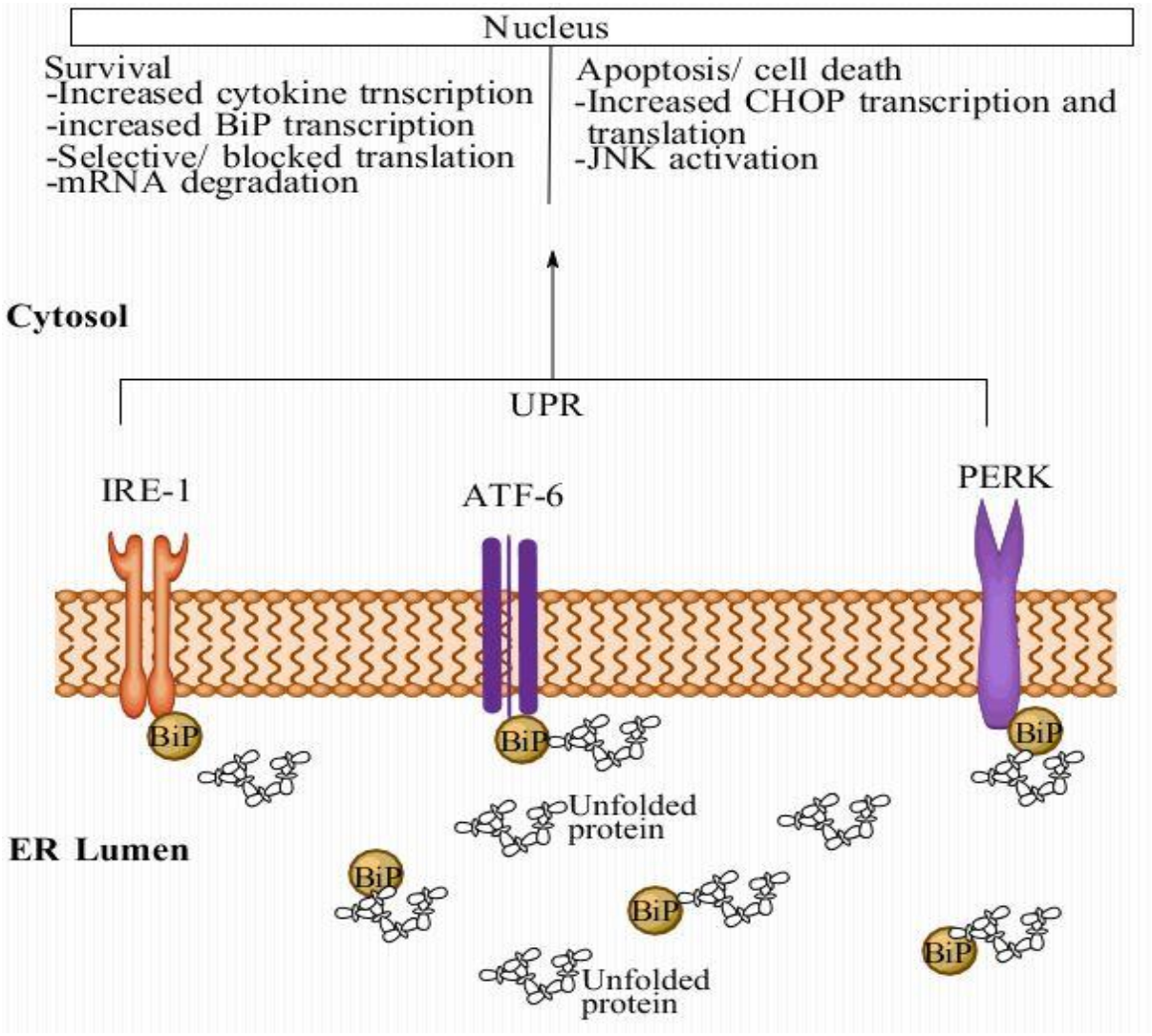

Figure 2.4: Unfolded Protein Response mediated by BiP. Activation of the ER membrane transducers leads to a cascade of kinases phosphorylation that either favour apoptosis through upregulation of CHOP and JNK activation. Similarly, cell survival can be favoured by blocking translation and degrading synthesized mRNA.

Even though BiP is natively an intracellular protein; it has been reported to be able of translocating to and expressed on cell membranes attached to peripheral proteins including both transmembrane proteins and external proteins such as glycosylphosphotidylinositol (GPI) and similarly secreted into the extracellular environment (Tsai et al., 2015) where it acts as an autoantigen and exert effects on immune cells through binding with surface receptors (Corrigall et al., 2004; Zhang et al., 2010). Over

the last years, its effect has been exploited in different immune cells including macrophages, dendritic cells, monocytes, T-cells and B-cells. BiP facilitates differentiation of mature dendritic cells to express anti-inflammatory phenotype through upregulation of Indoleamine-2,3-Dioxygenase (IDO) (Corrigall et al., 2009) and also stimulate myeloid cells to express an anti-inflammatory phenotype mostly observed in immature dendritic cells (Yang et al., 2016). In a study by Corrigall et al., (2004) BiP was shown to act on human peripheral blood mononuclear cells (PBMCs) to induce anti-inflammatory responses through upregulation of IL-10. It also affects the expression of CD86 and HLA-DR surface expression in monocytes which caused these antigen presenting cells (APCs) to be unable of activating T-cells. BiP effects also alter the ability of memory T-cells to recall antigens and initiate secondary responses. Immunological effects caused by BiP has ultimately established study platforms in areas relating to cancer, transplantation, and autoimmune studies (Brownlie et al., 2006; Yoshida et al., 2011). Contradicting results however were observed in *Giardia lamblia* infection in murine samples where recombinant BiP extracted from *Giardia lamblia* (rGlBiP) showed to promote pro-inflammatory responses through upregulation of CD80, CD86 and MHC II on mature dendritic cells leading to increased production of pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α (H.-Y. Lee et al., 2014). Another study by (Yoo et al., 2012)) showed that cell surface BiP expression can be upregulated by pro-inflammatory such as TNF-α an IL-1β. Additionally, this upregulation was associated with increased proliferation of synoviocyte cells and progressive pathogenesis of rheumatoid arthritis in synoviocytes. This suggested that cell surface BiP can modulate pro-inflammatory responses in synoviocytes. Interestingly, transfection of BiP gene into murine and human PBMC samples using a viral vector has been evaluated as having the same anti-inflammatory effects as stimulating with synthesized proteins in collagen arthritis inflammation (Shields et al., 2015). Supporting evidence of extracellular BiP binding to immune cells was shown in a study by Tang et al., (2016) where BiP induced the development of regulatory B-cells in murine samples and worked in synergy with CD40 to suppress proliferation of T-cells.

Binding of extracellular BiP with immune cells

BiP is a member of heat-shock proteins (Hsp70) that have been reported to have immune modulatory functions. Unlike other members of Hsp70 which are implicated in the induction of pro-inflammatory immune responses, BiP is reported to mostly facilitate anti-inflammatory responses. As a result, this chaperone has been studied in the induction of functional changes in immune cells (Bläss et al., 2001; Bodman-Smith et al., 2003; Corrigall et al., 2004). Secreted BiP binds to cell receptors as an autoantigen and causes functional changes. However, pathways involved in these processes are not fully elucidated (Figure 2.2), but it has been stated that most of the Hsp70 proteins are secreted through passive secretion. Studies on rheumatoid arthritis and cancer that have identified extracellular BiP observed that it is secreted without its anchor, a four amino acid sequence KDEL thus suggesting that it's not available as a result of membrane rupture or cell death (Corrigall et al., 2004). In particular, a study in murine collagen induced arthritis (CIA) revealed an IL-4 dependent effect of BiP on T cell responses which leads to secretion of anti-collagen specific cytokines including IL-5 and IL-10 (Brownlie et al., 2006). Even though BiP upregulation is beneficial to immune responses as it activates signal transducers that mediate cytokine and antibody gene expression, similarly, its downregulation has been associated with benefits in cancer suppression which could be a target of choice in studies such as B-cell lymphoma. Targeting this chaperone could be a promising approach that will aid in the development of new therapeutic procedures that will better control inflammation during diseases (Corrigall et al., 2009; Yang et al., 2016). Less data has been generated on binding of extracellular BiP onto immune cells since previous studies focused mainly on monitoring the cytokine profile in immune cell samples in the presence of BiP rather than assessing binding receptors or pathways involved during internalization. However, it has been suggested in a study by Becker et al., (2002) that free Hsp70 proteins bind immune cells, such as B-cells, through CD40 in an ATPase dependent manner. Even though this pathway has not been fully studied but BiP binding with immune cells such as B-cells was also shown by Tang et al., (2016) using fluorescently labeled BiP in a murine model. Binding and internalization of this chaperone protein on B-cell samples induced upregulation

of 3 populations of regulatory B-cells with distinct phenotypes (IL-10^{hi}, PDL-1, and FasL) and thus highlighting different regulatory mechanisms (Tang et al., 2016).

Regulatory function of mature/active regulatory B-cells

Initial studies on regulatory B-cells implied that these cells were only modulating immune responses until regulatory T-cell mature enough to take over the function. However, further studies elaborated on immune functions played by these cells using murine models and have repeatedly shown that regulatory B-cells exert their function mostly through IL-10 (Matsushita et al., 2008; Yanaba et al., 2009). Similar results have been obtained in human models, where both cytokines and cell surface receptors are implicated in the regulatory roles of these cells (Blair et al., 2010). Even though their development depends on various factors including the type of stimulant and the presence of micronutrients to drive immunometabolism; these cells have been shown to inhibit proliferation and function of macrophages, T helper 1 (Th1) and T helper 17 (Th17) cells while leaving T helper 2 (Th2) function unaffected (Flores-Borja et al., 2013). In various studies, this was confirmed by a decrease in secreted levels of cytokines such as Interferon gamma and Interleukin 17 (IL-17) while IL-4 levels were not affected by the presence of regulatory B-cells (Flores-Borja et al., 2013; Zhang et al., 2012). Regulatory B-cell importance is associated with maintaining immune balance after inflammatory responses in order to prevent autoimmune diseases progression by suppressing persisting T-cell responses (Yanaba et al., 2008). Cell-to-cell immunomodulatory function of regulatory B-cells occurs through the expression of ligands that target and bind to specific surface receptors expressed by other immune cells and this binding further enhance apoptotic pathways. Such immunomodulatory ligands include PDL-1 which targets PD-1 membrane protein on activated T-cells and monocytes; Fas-L which stimulate apoptosis of infected macrophages during chronic infections such as TB (Oddo et al., 1998; van Rensburg et al., 2017a, 2017b); and Glucocorticoid-Induced TNRF family-Related protein (GITR) which has been shown to be associated with negatively

modulating proliferation of regulatory T-cell during RA through binding to GITR-ligand expressed by these cells (Ray et al., 2012).

Immune responses driven by low or high frequencies of regulatory B-cells

In allograft and autoimmune diseases, high frequency of regulatory B-cells is associated with preventing progression into aggressive stages of inflammation. Functions carried by these cell subsets is highly similar to regulatory T-cells; however, these functions are believed to be alternating during inflammation in a sense that regulatory B-cells are mostly active during the onset of inflammation whereas regulatory T-cells come to action towards the end of inflammation. This notion has been supported in a variety of studies where regulatory B-cells were isolated in higher frequencies in healthy individuals but eventually depletes as inflammatory responses progress (Flores-Borja et al., 2013; Matsushita et al., 2008). The same "depletion-reappearing" trait of these cells has been observed in studies focusing on different inflammatory responses using both murine and human models. Recently, it has been observed in human samples studying B-cell immune responses during *M.tb* infection and this was believed to play a part in disease progression as their numbers vanished during active TB disease but upon treatment of the disease; these regulatory B-cells reappeared to a frequency equivalent to healthy individuals (van Rensburg et al., 2017b). Expression of regulatory traits in B-cells has been described in varying frequencies depending on B-cell development stage, location and type of inflammation. Highlighted in table 2.1 are recently reported frequencies of different regulatory B-cell populations in uninfected or healthy individuals and these ranges from 0.1% to as high as 12% of the total described B-cell population. However, *in-vitro* these frequency percentages could be stimulated to increase using different stimulants amongst of which include; LPS, TLR-9 agonist, and BGC (Blair et al., 2010; du Plessis et al., 2016a; van Rensburg and Loxton, 2018).

Table 2.1: Defined regulatory B-cell population frequencies in healthy human individuals expressing different surface markers and exhibiting diverse regulatory mechanisms.

Phenotype	**Species**	**Percentage (%)**	**Mechanism of suppression**	**Reference**
$CD24^{hi}CD27^{+}$	Human, PBMC	0.6	IL-10	(Iwata et al., 2011)
$CD24^{+}CD38^{+}$				
$CD24^{hi}CD38^{-}$		3.92	IL-10	
$CD24^{hi}CD38^{int}$	Human	3.71		(Blair et al., 2010)
$CD24^{hi}CD38^{hi}$		12.6		
$CD5^{+}CD1d^{+}IL10^{+}$	Human, PBMC	0.24±0.05	IL-10	(Chen et al., 2017)
$CD19^{+}IgM^{+}CD38^{+}$				
$Fas\text{-}L^{+}$	Human, PBMC	0.1-1.6	Fas-L	(van Rensburg et al., 2017a)
$PD1^{+}$				
$CD19^{+}IgM^{-}CD38^{+}$				
$Fas\text{-}L^{+}$	Human, PBMC	0.1-1.5	Fas-L	(van Rensburg et al., 2017a)
$PD1^{+}$				
$CD19^{+}PD\text{-}L1^{hi}$	Human, spleen	5.2	PD-L1	(Khan et al., 2015)
$CD19^{+}CD138^{+}CD27^{+}$	Human, PBMC	2.99	IL-10	(du Plessis et al., 2016)

Presence of regulatory B-cells and high secretion levels of IL-10 by these cells modulate differentiation of certain T-cell subsets expressing $CD4^{+}CD25^{-}$ T-cells to regulatory T-cells expressing FoxP3 which further mediate regulatory functions during inflammation (Flores-Borja et al., 2013). *In-vitro* work has shown that high levels of IL-10 are linked to impaired secretion and activity of certain pro-inflammatory cytokines such as TNF-α, IL-12, IL1-β, and IL-17; these are required during early inflammation to initiate and activate adaptive responses by recruiting T-cells and B-cells. However, in the context of *M.tb* infection, engagement of adaptive responses has been speculated to favour TB disease progression through the formation of granulomas which later becomes replication sites for the intracellular pathogen leading to more cells being infected within the structure as bacterial numbers grow and infected cells rapture through cellular necrosis and pyroptosis.

Conclusion and Future perspectives

Regulatory characteristic induction by BiP in B-cells

Less data is currently available on the induction of human regulatory B-cells using BiP indicating that more work still needs to be done to understand the pathways involved in the development of these cell types. Previous reports have shown that BiP has anti-inflammatory properties through induction of regulatory cells and this has been evaluated in different inflammatory studies with successful results (Bodman-Smith et al., 2003; Yoshida et al., 2011). Apart from inducing immune regulatory functions, its activity in immunoglobulin folding results in better antibody secretion which in turn facilitate better control of infectious pathogens (Fritz and Weaver, 2014). Its biological nature gives it an additional advantage by limiting non-specific inflammatory responses which may end up causing immuno-pathogenesis. Availability of this molecule in extracellular circulation and its autoantigen properties facilitate secretion of autoantibodies against it, this also displays an additional advantage since immune cells will efficiently opsonize and internalize it resulting in shorter time required for the development of regulatory traits. Induction of these traits; IL-10, Fas-L and PD-L1; in B-cells using BiP in a murine model study and its implication in inducing regulatory traits in T-

cells in human model studies have paved the way and indicated a high possibility of regulatory B-cell induction by this antigen in human settings (Tang et al., 2016). More studies must be conducted in the context of *M.tb* infection and BiP behaviour.

Regulatory B-cells as agents in therapeutic interventions

Inflammatory responses directed to intracellular pathogens such as *M.tb* faces challenges on directly eliminating the pathogen from inside the cell. This phenomenon results as pathogens such as M.*tb* have evolved a way to suppress destruction by proteolytic enzymes inside the phagosomes, additionally, antibodies and cytokines secreted towards this pathogen cannot penetrate cellular membranes to reach it, thus resulting in established infection and disease progression. Even though the formation of granulomatous structures during TB helps in containing the pathogen and preventing early spread; the pathogen may multiply to higher numbers and progress to active disease. Immune cell activity within the granuloma structure and upregulation of necrotic cytokines and perforins lead to the destruction of infected cells by necrosis which in turn leads to the release of more pathogens to infect other cells. Comparatively, regulatory B-cells has been suggested to induce apoptosis of infected cells by affecting cellular metabolism which in turn affect homeostasis of calcium, reactive oxygen species and nitric oxide thus affecting the survival of the intracellular pathogen. This prevents the presentation of antigens and activation of effector T-cells thus limiting activation of adaptive immune responses. Stimulating development or maintaining the frequency of these regulatory cells can present more benefits in host directed immunotherapies and control of intracellular pathogens likes *M.tb*.

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Chapter 3

The level of the endoplasmic reticulum stress chaperone protein - Binding Immunoglobulin Protein (BiP) decreases following successful TB treatment.

The work presented here has been formatted in the style of the International Journal of Infectious Disease to which it has been accepted for publication.

The level of the endoplasmic reticulum stress chaperone protein - Binding Immunoglobulin Protein (BiP) decreases following successful TB treatment.

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Keywords: TB treatment, M.*tb*, Binding Immunoglobulin Protein, Endoplasmic Reticulum stress

Word count: 3697

Abstract

Increased *Mycobacterium tuberculosis* (*M.tb*) burden inside the host leads to higher demand of response proteins. This in turn results in metabolic shift and cellular stress which is caused by the accumulation and trafficking of these proteins within the endoplasmic reticulum (ER). To resolve this, cells trigger Unfolded Protein Response (UPR) which is mainly mediated by Binding Immunoglobulin Protein (BiP)/ Glucose Regulated Protein 78 (GRP78) chaperone, which in turn upregulates its transcription. This chaperone protein facilitates proper protein folding within the ER, however, it can also be passively secreted to the extracellular environment or be expressed on cell surfaces attached to anchor proteins and transmembrane proteins. This notion has been shown in chronic inflammatory studies including cancer and arthritis detecting BiP specific antibodies from different sample types. This study analysed secreted BiP from plasma samples collected from healthy participants, newly diagnosed TB (TBdx) cases and over the course of TB treatment at week 1 (W1), month 2 (M2) and month 6 (M6). Our results revealed that during initial TB disease and treatment period, cells are subjected to stress conditions resulting in metabolic shifts which lead to secretion of intracellular UPR mediating chaperone protein, BiP. This was indicated by mean differences between TBdx (mean 40.88 ng/ml) and W1 (68.57 ng/ml) in TB participant groups. However, no difference was observed between the healthy group (mean 42.64 ng/ml) and TBdx (mean 40.88). Analyzing paired time point visits revealed increased BiP secretion during early TB treatment. Detection of BiP from plasma samples showed to decrease after successful TB treatment period to levels comparable to healthy control in our study groups. Evaluation of BiP levels in larger TB treatment studies may lead to the identification of a new target for early TB diagnosis and host directed immunotherapy.

Introduction

Binding Immunoglobulin Protein (BiP), also referred to as Glucose Regulated Protein 78 (GRP78), is an endoplasmic reticulum (ER) chaperone protein that plays a crucial role during protein synthesis by mediating proper protein folding through binding with polypeptide structures entering the ER lumen (Maddalo et al., 2012; Morris et al., 1997). Even though BiP interact with peptides, however,

its function is not required for extracellular secretion of proteins. This chaperone protein resides in the ER lumen, in an inactive state attached to three membrane transducer proteins (Inositol Requiring Enzyme-1 (IRE1), Protein kinase R-like Endoplasmic Reticulum kinase (PERK) and Activating Transcription factor-6 (ATF-6)). Activation of this chaperone occurs during conditions that pose cellular stress (Gass et al., 2002), specifically on ER, triggering Unfolded Protein Response (UPR) which has been reported as a cell survival mechanism. During this, a series of events may occur which may include termination of transcription and translation, destruction of unfolded or misfolded proteins through ubiquitination, and upregulation of GRP78/BiP expression (Rao and Bredesen, 2004). Even though this response is chiefly mediated by BiP/GRP78, it also requires the activity of the three signal transducers localized in ER membrane (IRE1, PERK and ATF-6) which phosphorylates cytosol kinases, and these metabolic pathways determine cell fate during UPR by either promoting cell survival or apoptosis. During UPR, BiP/GRP78 transcription is upregulated and this is mediated by the transcription factor YY1 which binds at the promoter region of the BiP gene. This transcription factor also acts as a cofactor for activation of ATF6 which is reported to be very essential during initiation of ER stress response (Baumeister et al., 2005). BiP upregulation has also been shown to be influenced by the activity of some pro-inflammatory cytokines, particularly TNF-α and IL-1β (Yoo et al., 2012). Research studies have also shown that during chronic inflammation and prolonged ER stress, BiP can translocate other cell compartments such as cell membrane where it can be attached to transmembrane proteins or glycosylphophatidylinositol (GPI) proteins. A study by Tsai et al., (2015) suggested that BiP can also escape ER lumen to other cell compartments attached to recently synthesized proteins. This could imply that during UPR, BiP may be secreted to the extracellular fluids through these similar process as it was shown that its substrate binding activity was more important in this regard (Tsai et al., 2015). Previous studies have reported on cell-free BiP in association with disease exacerbation, however, these focused on chronic diseases such as cancer and autoimmune disease (Bläss et al., 2001; Bodman-Smith et al., 2003; Zhang et al., 2010). However, mechanisms involved in secretion of this chaperone to extracellular fluids are not clearly

defined and it is also thought to escape during membrane repair by ER vesicles (Zhang et al., 2010). In an extracellular phase, this chaperone has been shown to act as an autoantigen on immune cells and leads to metabolic shifts which affect cell function (Bodman-Smith et al., 2003). Presence of BiP in extracellular fluids has been shown in different sample types including serum, plasma, and synovial fluid. Varying disease outcomes have been associated with extracellular BiP which shows to be both beneficial and detrimental. This chaperone protein was reported to be overexpressed in rheumatoid arthritis (RA) through detection of autoantibodies secreted specifically against it. During RA extracellular BiP was reported to upregulate anti-inflammatory responses which have been previously reported to be beneficial in resolving inflammation (Corrigall et al., 2004). Contradictory results were also reported where extracellular BiP was shown to enhance the progression of RA in synoviocyte by promoting proliferation of synoviocytes which in turn enhanced angiogenesis (Yoo et al., 2012). Additionally, the presence of this chaperone protein in the extracellular phase was reported to promote prognosis of RA by affecting the activity of cytotoxic T-cells (Bläss et al., 2001). Similarly, BiP pathogenesis has been reported during cancer inflammation and it is suggested that downregulation of BiP synthesis and activity can be beneficial in resolving cancer conditions by restricting synthesis of vital proteins required by the cancer cells (Schwarze and Rangnekar, 2010). Immunological response modifications associated with this chaperone protein led to research interests to evaluate its secretion during the anti-TB treatment period in infected participants. Previous immunological response studies highlighted dysfunctionality within B-cell subset in response to TB and this may be in part brought into play by the extracellular matrix. Extracellular BiP binding may lead to intracellular metabolic shifts that affect the expression of both soluble proteins and expression of cell surface proteins.

Method and Materials

Ethics statement

Ethical approval was obtained from the ethics committee of the Stellenbosch University Health Research Ethics Committee (N05/11/187) and the City of Cape Town City Health. The study was

conducted according to the Helsinki Declaration and International Conference of Harmonization guidelines. Written informed consent was obtained from all study participants.

Sample description

Blood samples were collected from participants recruited in the Sarepta area of Cape Town in the Western Cape region of South Africa. This included both male and female participants aged between 18-64 years old. Plasma samples were collected from a total of 81 participants; these included 42 male participants and 39 female participants. Collected samples were categorized into 2 major groups; healthy controls (32 participants) which had no indication of *M.tb* infection based on sputum culture, X-ray and Quantiferon release assay; and active TB disease group at diagnosis (TBDx) (29 participants) who had a positive sputum culture, X-ray with signs and symptoms suggestive of TB disease. TB cases were placed on treatment and followed up for three time points; week-1 (W1), month-2 (M2) and month-6 (M6). Collected plasma samples were then stored at -80^{o}C till analysis.

Enzyme Linked ImmunoSorbent Assay (ELISA) analysis

Collected plasma samples were recovered from the biorepository and analysed through ELISA. These samples included 32 healthy controls. TB disease group plasma samples included 29 baseline TB (diagnosis) with follow up time point visit of 8 participants in week-1, 7 participants in month-2, and 19 participants in month-6 follow-up visit, in addition, 20 new participants were recruited during M6 time point. Plasma samples were completely thawed at room temperature before ELISA analysis. These were then centrifuged at 1000 xg for 5 minutes to pellet any particulate material which might interfere with analyte binding. The Human GRP78 (Glucose Regulated Protein 78) ELISA kit from MyBioSource (MBS2533401, San Diego, USA), with a minimum sensitivity of 0.375 ng/ml and a detection range of 0.625-40 ng/ml was used as per manufacturer's instructions. The kit is specified to have no cross reactivity or interference between analogues and human GRP78. ELISA plates were read using the Bio-Rad iMark Microplate reader (California, USA) at a recommended wavelength of 450 nm.

Statistical Analysis

Obtained optical density (OD) readings were analysed using GraphPad PRISM 7 for Windows, GraphPad Software, (La Jolla, California USA, www.graphpad.com). BiP concentrations were analysed for differential expression between groups using the One-Way non-Parametric ANOVA, Mann Whitney t-test and unpaired *t*-test.

Results

BiP levels remain unchanged at diagnosis of active TB compared to healthy controls

In Figure 3.2 it is evident that even though BiP is an ER resident chaperone protein it can be secreted into plasma in small quantities even in healthy individuals. BiP secretion between participant groups showed a statistically significant difference (p=0.0270) with healthy group (mean 42.64 ng/ml) and TB diagnosis (mean 40.88 ng/ml) having comparable mean expression. BiP secretion was upregulated during early TB treatment (week 1) which showed the highest mean of 68.57 ng/ml. Furthermore, detection of BiP from these groups gradually decreased over the course of TB treatment approaching a healthy and TBdx baseline (Figure 3.2). In the second month of TB treatment (M2), BiP detection showed to decrease (mean= 60.92 ng/ml) and this showed further decrease by month 6 (M6) as indicated by mean of 37.42 ng/ml. In contrast, no statistically significant difference was observed between healthy, TB diagnosis, month 2 and Month 6.

TB treatment on follow up participants between TBdx and W1

BiP upregulation was further illustrated in TB treatment follow-up participant pairs (Figure 3.3 and Table 3.1) which indicate direct upregulation of this chaperone from respective individuals with detection range between 30.60 ng/ml–65.57 ng/ml (TBdx) and 60.18 ng/ml–86.77 ng/ml during week 1 (W1) anti-TB treatment. This showed a significant difference (p = 0.0078) in BiP secretion between these two time point visits.

BiP levels remain unchanged from week 1 of TB treatment till the end of therapy

Participant follow-up visits at month 2 anti-TB treatment showed no significant change in extracellular BiP detection (Figure 3.4) with their respective diagnosis time point with p= 0.1563 and a detection range between 58.41 ng/ml – 64.80 ng/ml as shown in Table 3.1. BiP detection at the end of anti-TB treatment (month 6) indicated a detection range between 56.64 ng/ml and 73.45 ng/ml; this showed no significant difference with BiP detection during TBdx (p = 0.4800) (Table 1).

Discussion

In this study, we have provided evidence that during TB disease treatment, Unfolded Protein Response (UPR) is activated leading to upregulation of intracellular mediators such as BiP which in turn escapes to extracellular phase. Activation of UPR is regarded as a survival mechanism which cells utilize to cope with a high demand for response proteins during inflammation (Lin et al., 2007). *Mycobacterium tuberculosis* infection is classified into different infections stages with latently infected individuals (LTBI) at the early stage of infection where the bacterium is thought to be contained (Flynn and Chan, 2001). During this time, immune cells are subjected to minimal metabolic shift resulting in minimal stress response activation; this is evident in our results by non-significant different in BiP secretion levels between healthy participants and those diagnosed as positive for M.*tb* infection through sputum cultures, interferon gamma release assay (IGRA) and X-ray results. This finding compares with what has been shown in other studies done in cancer and arthritis showing that during prolonged stress conditions, cells activate UPR response resulting in secretion of intracellular ER chaperone proteins amongst which include BiP (Bodman-Smith et al., 2003; Zhang et al., 2005). Even though mechanisms involved in this are not fully elucidated, it has been suggested that cells elude these intracellular components through passive mechanisms during ER vesicle translocation or they can also be released in conjunction with their protein substrates (Tsai et al., 2015). *M.tb* has been shown to be able to manipulate and avoid innate immune responses and multiply within the host (Raghuvanshi et al., 2010; Sia et al., 2015), this leads to the increased bacterial burden which may eventually activate UPR due to the high demand of immunoglobulin and metabolic shift. This leads

to secretion of these mediators to the extracellular phase where they affect disease outcome. However, pharmacological agents have also been implicated in activating UPR (Srinivasan et al., 2005). This may suggest that TB treatment regimens also participate in upregulation of UPR as they need to be metabolized by cells, mainly liver cells, into inactive form before being eliminated from the body. This results in metabolic shifts within the cells which forces them to upregulate transcription and synthesis of specific protein compounds to mitigate the condition, these including BiP. The latter phenomenon can be correlated with the high secretion levels (Figure 3.2) of this stress response protein during the initial treatment period which is also characterized by a sharp decline in bacterial numbers. Even though BiP is upregulated during initial metabolic shifts (Bodman-Smith et al., 2004); transcription and protein synthesis become stabilized with unchanged conditions or decline with resolved inflammation. Theoretically, during TB treatment period, the decrease in bacterial burden over time should result in decreased metabolic demand from activated immune cells and this correlated with our findings where extracellular BiP showed to stabilize and gradually decrease after week 1 into TB treatment. Similarly, the switch of TB drugs after month 2 (from 4 drugs to two) and decreased bacterial burden does not facilitate further upregulation of UPR also because apart from other TB regimen rifampicin is not metabolized in the body suggesting even laser protein demand. Even though activation of UPR is considered a cell survival mechanism, it has also been shown that prolonged response triggers cell death through apoptotic pathways by upregulation of the transcription factor C/EBP homologous protein (CHOP) (Mozos et al., 2011). The latter can be beneficial for infection control since TB disease is caused by an intracellular pathogen and inducing apoptosis results in cell death from within by limiting metabolites flux which are vital for *M.tb* survival. Apoptotic pathways are known to prevent early cell membrane rapture thus limiting the spread of *M.tb* to uninfected cells. This is opposite to necrotic pathways which have been implicated to be utilized by the pathogen in question to infect and reside in other immune cells. Upregulation of BiP in the cellular microenvironment has been shown to induce the anti-inflammatory response in immune cells either by upregulation of IL-10 synthesis (Yoshida et al., 2011) or upregulation of

surface markers involved in programmed cell death (Tang et al., 2016). Our study, evaluating the utility of secreted BiP over the course of TB treatment must be seen as an initial assessment as the number of participants with active TB were relatively low. Even though BiP levels were detected with a standard methods, however, protein degradation might have occurred over time resulting in lower levels of this respective biomarker. Future studies must evaluate BiP levels from a larger group of freshly isolated plasma and serum samples from participants at different *M.tb* infection stages.

Acknowledgement

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Figures

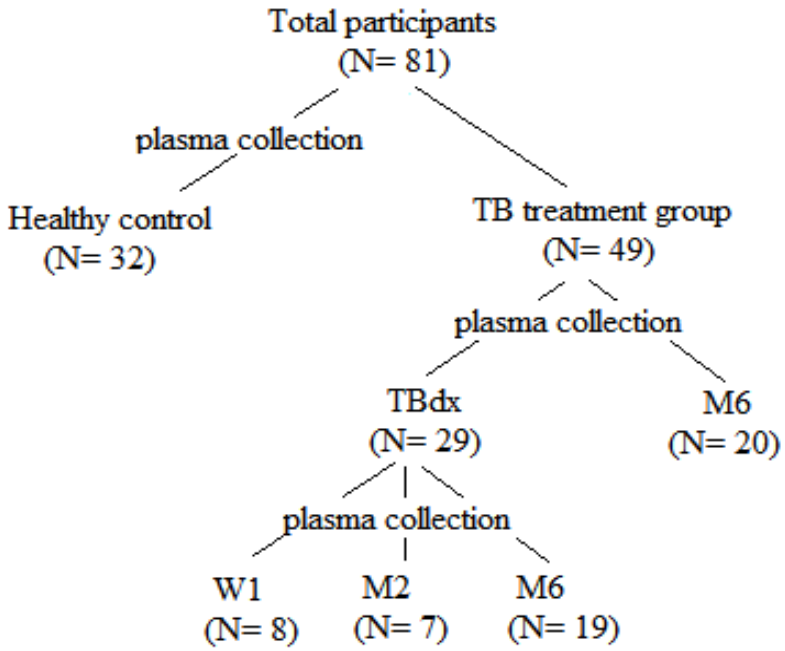

Figure 3.1: Plasma sample collection at specific time points. N= total number, TB=tuberculosis, TBdx= TB diagnosis, W1= week-1, M2= month-2 and M6= month-6

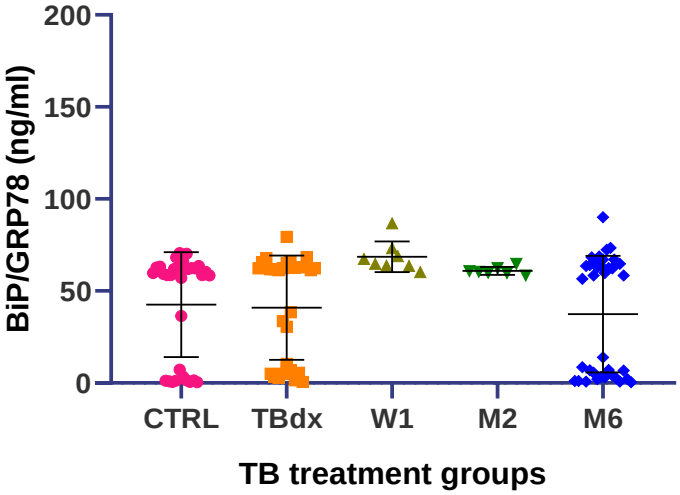

Figure 3.2: Detection of secreted Binding Immunoglobulin Protein (BiP) during TB treatment and healthy states. Statistical difference analysis through nonparametric one-way ANOVA, p= 0.0270; where CTRL=healthy group, TBdx=TB treatment group at diagnosis, W1=week 1 treatment group,

M2=month 2 treatment group and M6=month 6 treatment group; error bars show mean with standard deviation

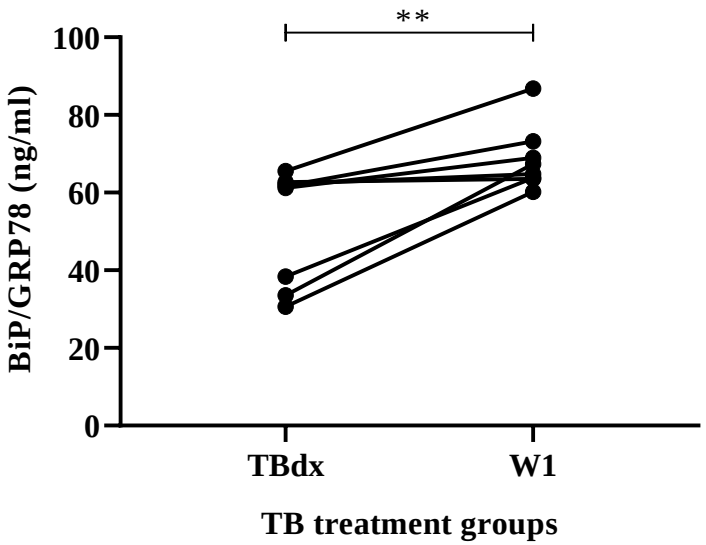

Figure 3.3: Increased levels of BiP following one week of TB treatment. Sample pairs analysed for changes in BiP secretion one week after the start of TB treatment. Statistical difference assessed through nonparametric paired *t*-test (Wilcoxon matched pairs test) $p = 0.0078$, where ** = $p<0.01$

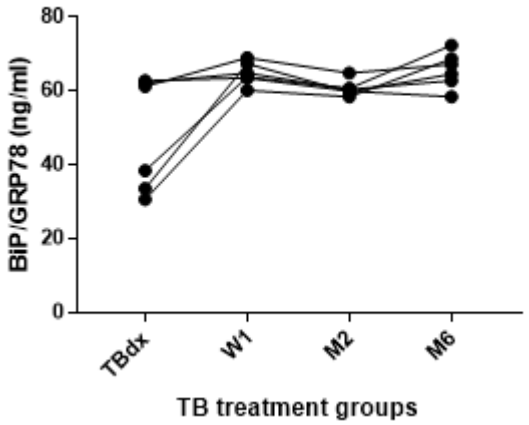

Figure 3.4: Paired time point visits during TB treatment revealed increased secretion of BiP by TB treatment groups. Statistical difference assessed through nonparametric paired Friedman test, $p = 0.0057$.

Tables

Table 3.1: TB treatment follow-up participants; TBdx = TB diagnosis, W1= week 1 of TB treatment, M2 = month 2 of TB treatment, M6 = month 6 of TB treatment, N= number of participants, LB = lower bound and UP = upper bound.

Variable	Treatment Groups	N	Mean Levels (ng/ml)	Standard deviation	p-value	95% CI for the mean		Min (ng/ml)	Max (ng/ml)
						LB	UB		
BiP	TBdx	8	52	14.95	0.0078	36.5	64.5	30.60	65.57
	W1	8	67.91	8.78		60.57	75.25	60.18	86.77
	TDdx	7	50.06	15.03	0.1563	36.16	63.96	30.60	62.74
	M2	7	60.92	2.10		58.98	62.86	58.41	64.80
	TBdx	19	59.50	12.90	0.4800	52.87	66.13	30.60	79.36
	M6	19	64.59	4.43		62.31	66.87	56.64	73.45

Chapter 4

Binding immunoglobulin protein induces anti-inflammatory responses and a killer phenotype in B-cells during TB disease.

This work has been formatted as a manuscript ready for submission

Binding immunoglobulin protein induces anti-inflammatory responses and a killer phenotype in B-cells during TB disease.

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Keywords: Binding Immunoglobulin Protein (BiP), Mycobacterium tuberculosis (*M.tb*), regulatory/killer B-cells, pro- and anti-inflammation.

Word count: 7043

Abstract

Mycobacterium tuberculosis (*M.tb*) infection remains a threat with a high mortality rate worldwide despite the availability of anti-tuberculosis (TB) treatment. This highlight the importance of establishing new measures to mitigate the infection. Host directed therapies (HDT) represent a promising platform through the discovery of novel host biomarkers and induction of immune cell subpopulations that will aid in better control of TB disease. Regulatory B-cells are continuously implicated in resolving chronic inflammation and has been isolated and shown to regulate responses through various mechanisms including secretion of anti-inflammatory cytokines and expression of cell surface ligands such as IL-10, IL-35, TGF-β, granzyme-B, Fas-L, PD-L1 and FoxP3. Previous studies have shown that these B-cell characteristics can be induced by different antigenic material during bacterial and viral infections. Here, we explored the ability of Binding Immunoglobulin Protein (BiP) to induce a regulatory B-cell phenotype *in vitro*, from mycobacterium tuberculosis (*M.tb*) exposed and unexposed healthy participants. We evaluated cell surface expression and secreted cytokine profile from 8 stimulation conditions, including unstimulated and stimulated with antigens: human recombinant BiP full-length protein (20ug/ml), H37Rv (MOI: 1:10), TLR-9a (0.5 µM), INH (1 µM), BiP plus TLR-9a, pooled BAL at TB Diagnosis (TBdx) and month 6 (M6) TB treatment. We found that BiP upregulated the co-expression of Fas-L (CD178) and IL5Rα (CD125) in both healthy (p=0.0147) and *M.tb* infected participants (LTBI) (0.0216). This chaperone showed synergy with TLR-9a to further upregulate the co-expression in both healthy (0.0002) and LTBI (p=0.0015). However, the cytokine profile in BiP stimulated samples showed a significant decrease in Interleukin (IL)-10 (QFN positive p=0.0350); conversely, increased soluble Fas-L (sFasL) (p=0.0402), IL-4 (QFN negative p=0.0006; QFN positive p=0.0043), IL-5 (QFN positive p=0.0002) and IL-13 (QFN negative p=<0.0001; QFN positive p=0.0006). H37Rv resulted in elevated levels of IL-1β (p=0.0001) in quantiferon positive group, Tumor Necrosis Factor-alpha (TNF-α) (QFN negative p=0.0334; QFN positive p=0.0114) and IL-10 (QFN negative p=0.0385; QFN positive p=0.0004). Intracellular pathways showed increased activation of p70S6K upon exposure to H37Rv.

In addition, the QFN positive group had increased activation of IRS1 (0.0179) and p70S6K compared to the QFN negative group. Our study highlights the effects of BiP on cell functionality which in future could be harnessed as host directed therapy against TB disease.

Introduction

Mycobacterium tuberculosis (*M.tb*) infection is listed among the deadly diseases worldwide. The World Health Organisation estimated about 10.4 million cases of TB disease in 2016 and even with a 3% drop in the global TB rate each year (WHO, 2017), there is still a need to advance early detection of this disease and therapeutic measures. Although TB treatment is readily available, host directed immunotherapies (HDT) are becoming a promising adjunct platform for better control of TB disease (Hawn et al., 2013; Tobin, 2015). This involves targeting different aspects of the host immune system including immune response mediating cells and their secreted biomarkers (Tobin, 2015). *In vitro* studies have indicated that immune cells respond differently to varying antigens and that this can elicit either pro-inflammatory or anti-inflammatory responses (du Plessis et al., 2016; van Rensburg and Loxton, 2018). A subpopulation of B-cells with regulatory/killer phenotype (B-regs) was studied by van Rensburg et al., (2017) during the TB treatment period. However, their function during this chronic disease is unknown as they become dysfunctional over the course of TB treatment and re-establish their frequencies after the successful treatment phase. Interleukin (IL)-10 secretion is regarded as the hallmark of regulatory B-cell (Horikawa et al., 2013), however, studies have shown that the regulatory functions of B-cells can equally be mediated through the expression of other cytokines such as IL-35 (Shen et al., 2014), Transforming growth factor beta (TGF-β) (Guo et al., 2015; Lee et al., 2014) and to a lesser extent, expression of FoxP3 (Guo et al., 2015; Noh et al., 2010). This B-cell subpopulation is suggested to develop at any stage during B-cell development, even though the mechanisms involved have not been fully characterized (Rosser and Mauri, 2015). Development of regulatory phenotypes in B-cells have been shown to be induced by varying factors including engagement of Toll-Like Receptor (TLR) molecules (TLR 2, 4, 9), viral and bacterial

infection, ER stress chaperones, LPS and soluble egg antigens (SEA's) (LENERT et al., 2005; Neves et al., 2010; Wang et al., 2017). During tuberculosis disease, the presence of regulatory B-cells showed to alter the function of Th17 cells while leaving T-helper 1 (Th1) cells unaffected suggesting a skewed balance between these two pro-inflammatory mediators (Torrado and Cooper, 2010; Zhang et al., 2012). As a result, recent studies have taken interest to the non-humoral functions of B-cells during inflammation and evidence suggest a growing platform of a killer/regulatory B-cells studies during different chronic inflammatory responses (Mauri and Menon, 2015). Killer (Fas-L^+) and regulatory phenotype (IL-10) of B-cells was shown to be inducible *in vitro* by various antigens including BCG (van Rensburg and Loxton, 2018), LPS (Yanaba et al., 2008) and TLR-9a (du Plessis et al., 2016) in the context of tuberculosis and other chronic diseases. These regulatory functions are observed within the $CD5^+$ B-cells and $CD24^+CD38^+$ B-cells during (van Rensburg and Loxton, 2018; Zhang et al., 2012, 2014). In a recent study by Tang et al., (2016) an endoplasmic reticulum (ER) chaperone protein, Binding Immunoglobulin Protein (BiP), was shown to induce elevated levels of IL10 in isolated murine B-cells with upregulated expression of death inducing ligands; Fas-L and PD-L1. BiP is an ER chaperone protein residing in the lumen in an inactive form attached to three membrane transducers. This chaperone protein has been shown to escape the ER through various mechanisms including passive translocation with recently folded proteins and translocation with cell membrane proteins (Tsai et al., 2015). Its upregulation has been reported to be induced by stress conditions and pro-inflammatory cytokines such as IL-1β and TNF-α (Yoo et al., 2012) When exposed or secreted to the extracellular space it act as an autoantigen which bind and drive immune cell responses mainly towards anti-inflammatory responses (Bodman-Smith et al., 2003; Corrigall et al., 2009, 2004; Tang et al., 2016; Yoshida et al., 2011). In this study, we aim to evaluate the ability of BiP to drive anti-inflammatory responses and induce a regulatory/ killer phenotype in B-cells during exposure/infection with *M.tb*.

Material and Methods

Ethics statement

Ethical approval was obtained from the Stellenbosch University Health Research Ethics Committee (N16/05/070). All participants gave written informed consent before participating in the study.

Participant description

A total of 20 participants were recruited for this study who were all HIV negative. As illustrated in Table 4.1, these included 12 healthy participants with no traces of previous exposure to *M.tb* (QFN negative) and 8 latent TB infected (LTBI) individuals (QFN positive) as indicated by the Interferon Gamma Release Assay (IGRA) and chest X-ray. Study participant's age ranged between 24 and 58 years old with a Mean ± SD of 32.1 ± 9.2.

Table 4.1: Participant clinical and demographic information

Participant Groups	Gender		N	Mean age ± SD
	Male	Female		
Total (N) Participants	8	12	20	32.1 ± 9.2.
QFN Negative	4	8	12	29.5 ± 6.0
QFN Positive	4	4	8	36.0 ± 12.1

QFN= QuantiFeron, N= participant number, SD= standard deviation

Peripheral blood mononuclear (PBMC) isolation and processing

Sodium heparin tubes were drawn for PBMC isolation, using a Ficoll Paque (GE Healthcare Life Sciences) gradient method. Isolated PBMCs were cryopreserved in 10% dimethylsulfoxide (DMSO) and stored at -80^{o}C in a Mr Frosty overnight after which it was transferred to liquid nitrogen (LN) until further experimental work.

PBMC overnight stimulation

Cells were thawed and the viability by trypan blue exclusion method ranged between 93% and 100%. Approximately 1 x 10^6 cells/well were dispensed into a 96 well culture plate for each stimulation condition. Samples were stimulated in a Biosafety level-3 facility under the following conditions: unstimulated PBMC, BiP (20 µg/ml, SPR 119A, StressMarq Biosciences inc, Canada), Toll-like Receptor-9 agonist (TLR-9a) (0.5 µM, Miltenyi Biotech, USA), Mycobacterium tuberculosis Strain H37Rv (MOI 1:10), Isoniazid (INH) (1 µM, Sigma-Aldrich cat:55-85-3, South Africa), BiP+TLR-9a (respective concentrations), pooled broncho-alveolar lavage (BAL) fluid from untreated TB at diagnosis (TBdx) and pooled BAL at month 6 (M6) of TB treatment. Culture plates were incubated at 37^{o}C (5% CO_2) for 24 hours after which culture supernatants were collected and stored at -80^{o}C. The cell pellet was fixed, cryopreserved and stored in LN till further analysis.

Multi-cytokine and kinase analysis

Luminex multiplex assay (magnetic bead based) was used for measuring cytokine secretion in culture supernatants. These included: IL-1β, IL-4, IL-5, IL-10, IL-13, TNF-α, granzyme B, Fas-L (R&D systems cat: LXSAHM-08, USA) TGF-β1, TGF-β2 and TGF-β3 (Merck cat: TGFBMAG-64K-03, USA). Plates were analysed using the MAGpix (Bio-Rad, USA) and Bioplex200 (Bio-Rad, USA) instruments.

a) Kinase activity multiplex

Stimulated cells were recovered from LN and split into two for all stimulation conditions with ~0.5 x 10^6 cells used for kinase activity and the remaining cells used for flow cytometry analysis. Intracellular kinases were measured for respective stimulation conditions and these included: Akt, mTOR, GSK3α, IGF1R, IR, IRS1, p70S6K, PTEN, RPS6 and TSC2 (Merck cat: 48-612MAG, USA). Stimulated PBMC were lysed with provided lysis buffer in the presence of a freshly prepared protease inhibitor cocktail (Merck cat: 535140-1SET, USA). The lysates were filtered through 0.65 µm low protein binding Durapore PVDF filters (Merck cat: UFC40DV25, USA) and analysed.

Flow cytometric analysis of B- and T-cell lymphocytes

Commercially available monoclonal antibodies were used to measure cell surface expression of CD3-PerCP, CD5-APC-CY7, CD19-BV510, CD38-PE-CY7, CD125-PE, CD178-APC, CD279-BV421, IgM-FITC (all antibodies supplied by BD). Figure 4.1 and 4.2 shows the gating strategy to identify killer B-cells and death receptors on T-cells. Fluorescence-Minus-One (FMO) controls were used to determine gating cut-off.

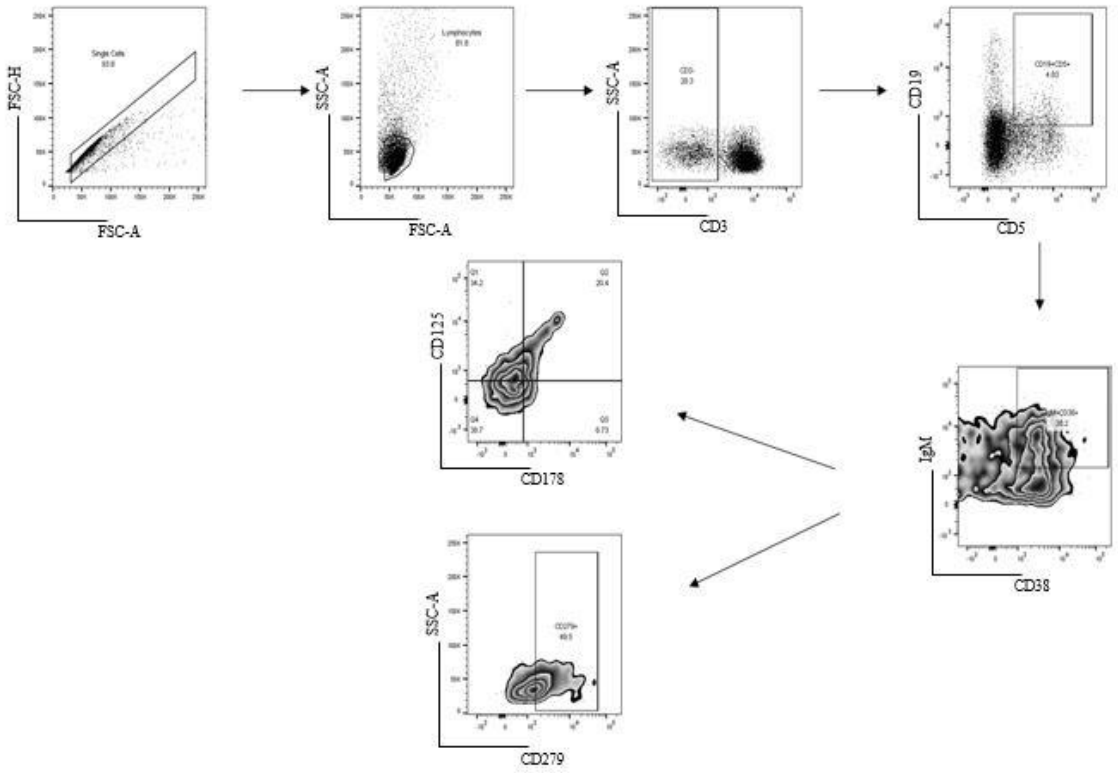


Figure 4.1: Gating strategy to determine killer B-cells isolated from human PBMC. Activated killer B cells were identified as $CD19^{+}CD5^{+}$ $CD38^{+}IgM^{+}$ B-cells co-expressing CD178 (Fas-L) and CD125 (IL-5Rα). The expression of CD279 (PD-1) was measured within the IgM+CD38+ B-cell population.

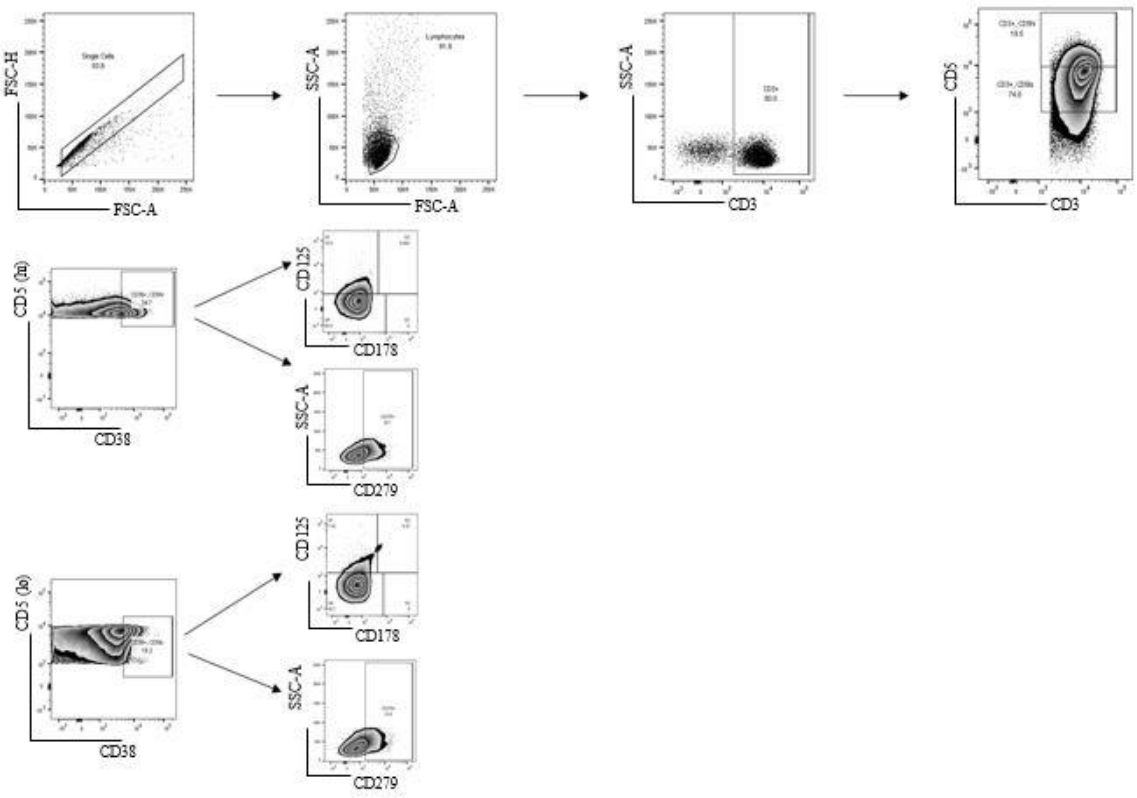


Figure 4.2: Gating strategy followed to assess phenotypic changes within $CD3^{+}CD5^{(lo)}$ and $CD3^{+}CD5^{(hi)}$ activated T-cells on expression of FasL, PD-1 and IL-5Rα.

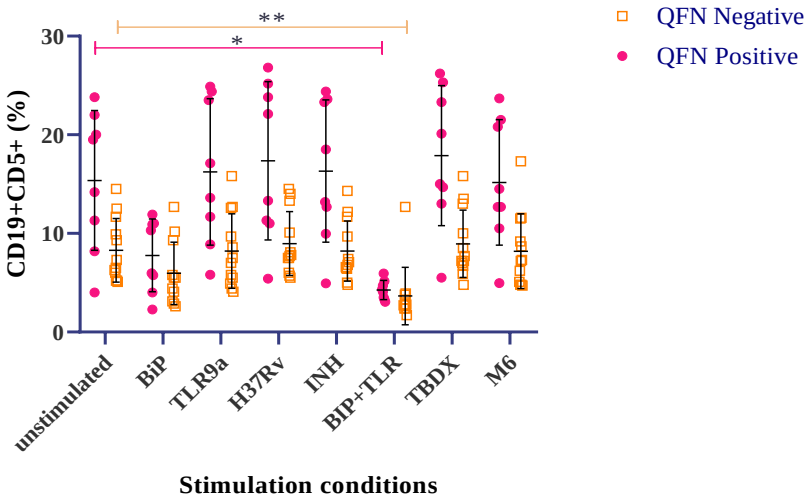
Data analysis

Statistical analysis was performed in Statistica 13 (TIBCO Software inc, USA) using Variance Estimation and Precession (VEPAC) to test for significant differences in cytokine secretion. Flow cytometry analysis was done using Flowjo software version 10 (Treestar) (Flowjo lcc, USA) and statistical difference between cell population frequencies evaluated using GraphPad prism 8 software (GraphPad, USA), using multiple *t*-test for differences between groups and two-way ANOVA for differences between stimulation conditions within each group. Kinase activity results were analysed using GraphPad prism 8 software (GraphPad, USA), using a two-way ANOVA.

Results

Expression of killer phenotype in CD19+CD5+ B-cell population

Comparison of surface maker expression between Quantiferon (QFN) negative and positive groups was achieved through multiple *t*-test. QFN positive group showed elevated frequencies of $CD19^{+}CD5^{+}$ B-cell population compared to QFN negative group (presented in Figure 4.3); significant difference was obtained between unstimulated PBMC's (p=0.0069) and PBMCs stimulated with TLR-9a (p=0.0049), H37Rv (p=0.0042), INH (p=0.0026), BAL TBdx (p=0.0014) and M6 (p=0.0064). Furthermore, this frequency was significantly decreased by BiP plus TLR-9a stimulation within both participant groups (QFN negative p=0.0030 and QFN positive p=0.0151) compared to their respective unstimulated PBMCs. Activation of mature $CD19^{+}CD5^{+}$ B-cells as indicated by expression of $CD38^{+}IgM^{+}$ indicated significant upregulation by TBdx stimulation within both participant groups (QFN negative p=0.0001 and QFN positive p=0.0098). IL-5Ra (CD125) expressing cells within $CD19^{+}CD5^{+}CD38^{+}IgM^{+}$ were down regulated by BiP (p=0.0173) and BiP plus TLR-9a (p=0.0051) within QFN negative group. In contrast, this population was upregulated by TLR-9a stimulation in QFN positive group (p=0.0071). Additionally, IL-5Rα expression was also increased by H37Rv (QFN negative p=0.0111 and QFN positive p=0.0157) and TB dx (QFN negative p=0.0044 and QFN positive p=0.0067) stimulations within both participant groups compared to their respective unstimulated samples. Co-expression of Fas-L (CD178) within $CD19^{+}CD5^{+}CD38^{+}IgM^{+}IL5Ra^{+}$ B-cell population was significantly upregulated by BiP (p=0.0216), BiP plus TLR-9a (p=0.0015) and TBdx (p=0.0051) within QFN positive group and similarly within QFN negative group (BIP p=0.0147, BiP plus TLR-9a p=0.0002 and TBdx p=0.0014) compared to their respective unstimulated samples. PD-1 ($CD279^{+}$) expression within $CD19^{+}CD5^{+}CD38^{+}IgM^{+}$ cell population was significantly upregulated by TLR-9a (p=0.0372) in QFN-negative group and H37Rv (p=0.0327) in QFN positive group compared to the unstimulated samples.



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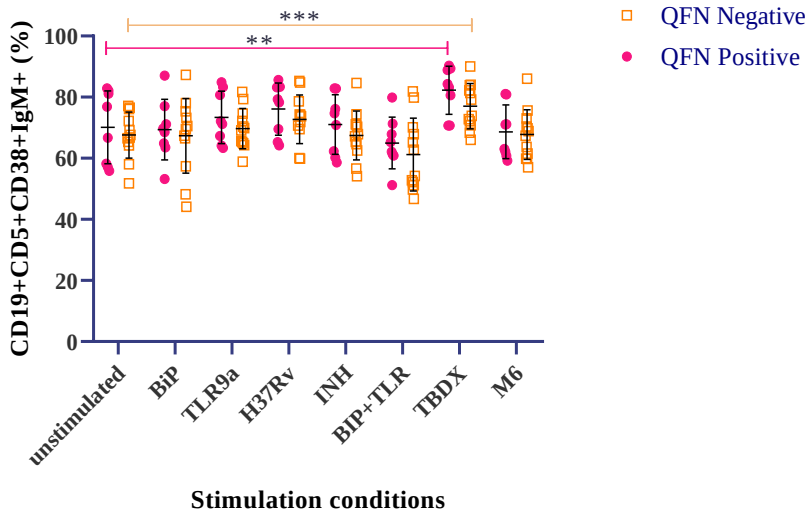

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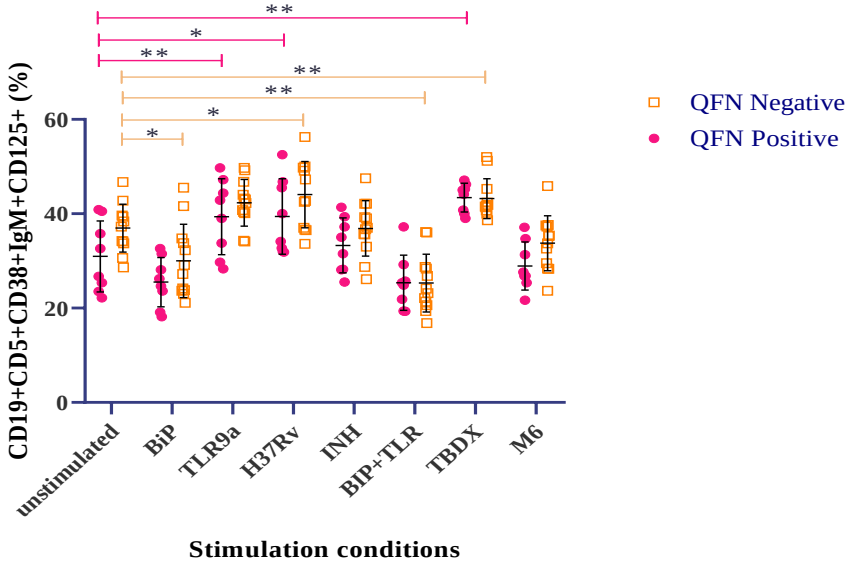

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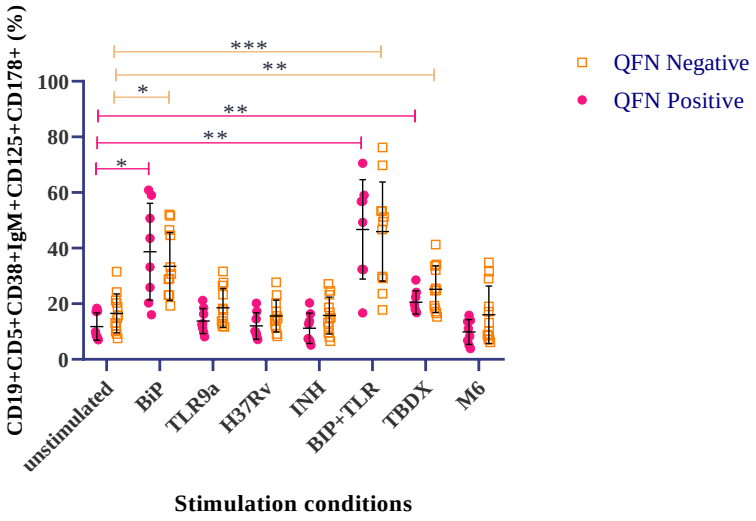

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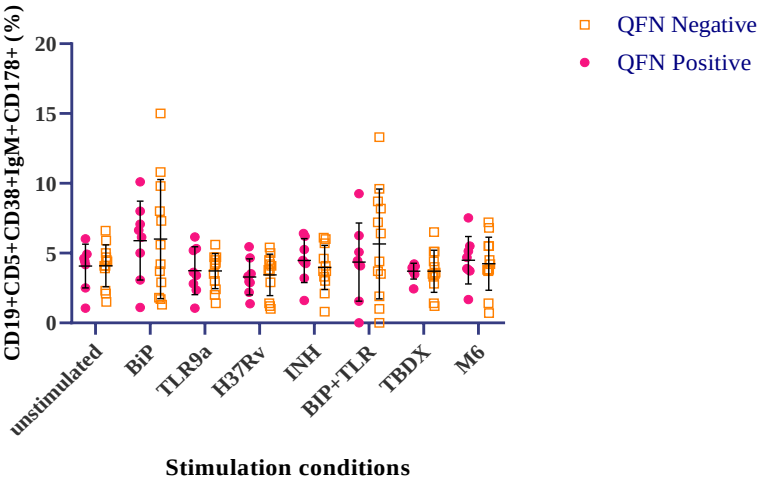

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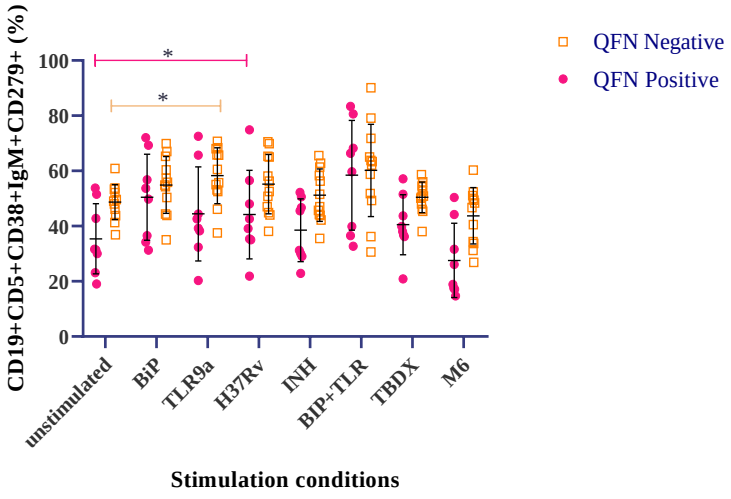


f)

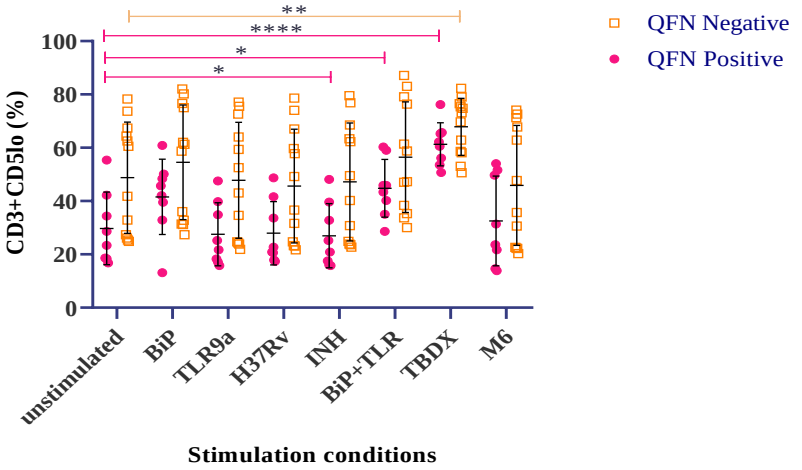
Figure 4.3: Cell surface immunoglobulin expression within $CD5^+$ B-cells, where B-cell subpopulation frequencies are represented as a) $CD19^+CD5^+$, b) $CD19^+CD5^+CD38^+IgM^+$, c)

$CD19^+CD5^+CD38^+IgM^+CD125^+$, d) $CD19^+CD5^+CD38^+IgM^+CD125^+CD178^+$, e) $CD19^+CD5^+CD38^+IgM^+CD178^+$, f) $CD19^+CD5^+CD38^+$ IgM^+CD279^+. Statistical significance was tested using non-parametric multiple *t*-test for differences between participant groups, respective to stimulation conditions, and non-parametric Two-way ANOVA for differences between stimulation conditions within each group. Significant difference is indicated by asterisk where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001.

Cell surface expression within CD3+CD5+ T-cells.

a) CD5 (lo) T cell receptor expression

Our results showed significant upregulation (Figure 4.4) of $CD3^+CD5^{lo}$ expressing T-cells after stimulation with INH (p=0.0322), BiP plus TLR-9a (p=0.0178) and BAL (TBdx) (p=<0.0001) within QFN positive participants whereas within QFN negative this was only upregulated by BAL (TBdx) (p=0.0039). No significant difference was obtained in the frequency of this cell population between participant groups. Activation (CD38 expression) within $CD3^+CD5^{lo}$ population was significantly upregulated by BAL TBdx in QFN positive group (p=0.0032). However, this activation was significantly downregulated by BiP plus TLR-9a within both participant groups (QFN negative p=0.0028 and QFN positive p=0.0032). Expression of IL-5Rα within $CD3^+CD5^{lo}CD38^+$ cell population was significantly upregulated by BiP stimulation (p=0.0386) in QFN negative; in addition, this was upregulated by BiP plus TLR-9a (QFN negative p=0.0118; QFN positive p=0.0057) and BAL TBdx (QFN negative p=0.0008; QFN positive p=<0.0001) within both participant groups compared to the unstimulated cells. Significant increase of PD-1 (CD279) expression within $CD3^+CD5^{lo}CD38^+$ was observed in BAL TBdx stimulation in both QFN negative (p=0.0017) and QFN positive (p=0.0290) groups compared to their respective unstimulated samples. Additionally, the expression of this receptor was significant in QFN positive compared to QFN negative group (p=0.0023). Fas-L single expression was not detected on these cells, however, co-expression with IL-5Rα was detected at lower frequencies between groups and stimulation within each group (not significant).



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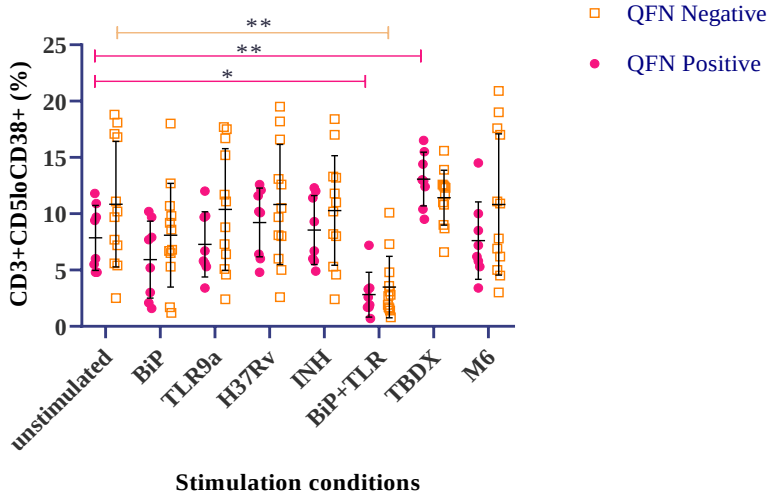

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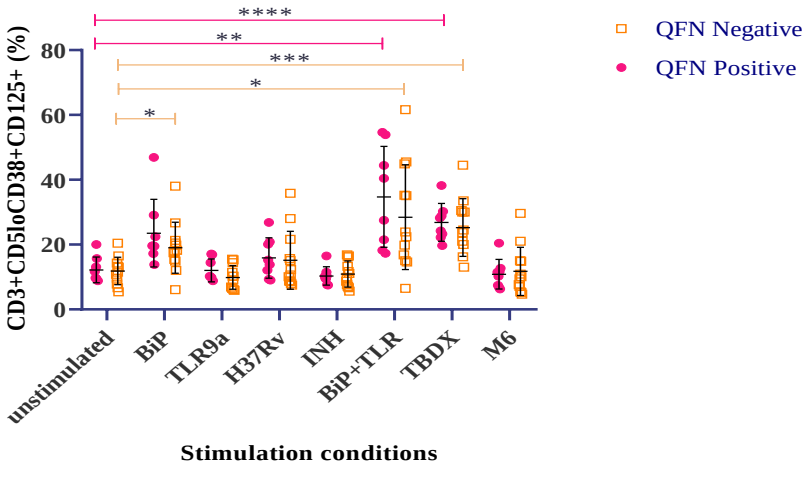

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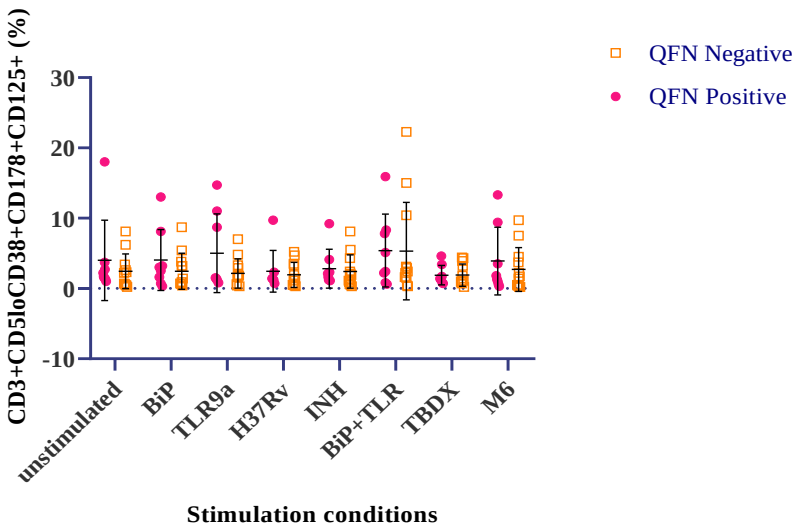

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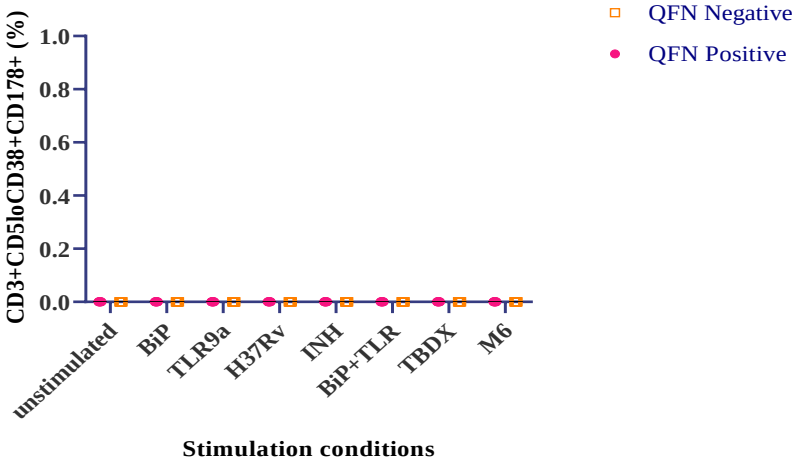

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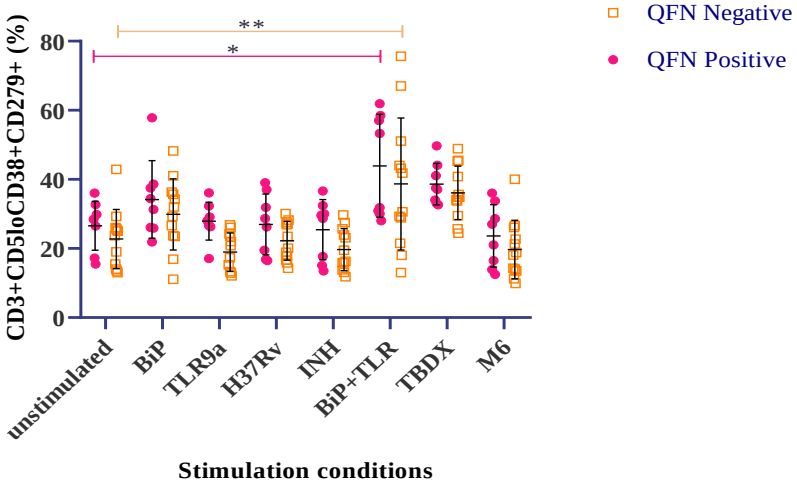


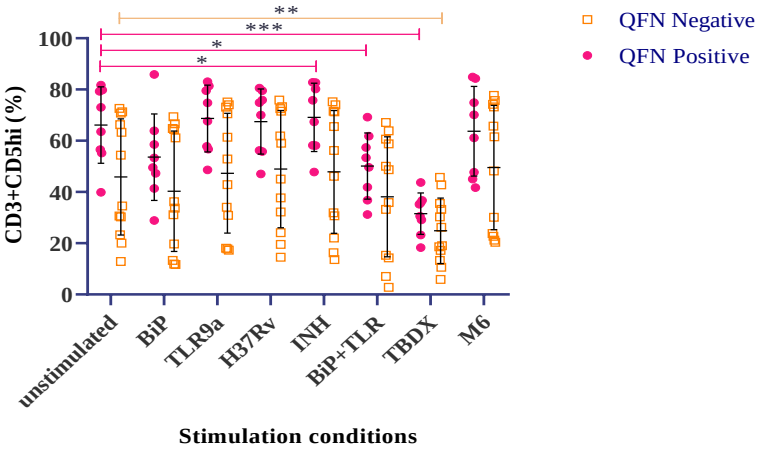
f)

Figure 4.4: Cell surface expression within T-cell subpopulations, where cell frequencies are represented as a) $CD3^{+}CD5^{lo}$, b) $CD3^{+}CD5^{lo}CD38^{+}$, c) $CD3^{+}CD5^{lo}CD38^{+}$, d)

$CD3^+CD5^{lo}CD38^+CD125^+CD178^+$, e) $CD3^+CD5^{lo}CD38^+CD178^+$ and f) $CD3^+CD5^{lo}CD38^+CD279^+$. Significant differences were tested through multiple *t*-test between participant groups and two-way ANOVA between unstimulated PBMC and other stimulation conditions within each group. Significant difference is indicated by asterisk where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001.

b) CD5 (hi) T cells surface marker expression

Comparison between unstimulated and stimulated PBMC within each group (Figure 4.5), showed upregulated frequency of $CD3^+CD5^{hi}$ T-cells by stimulation with INH (p=0.0300), BiP plus TLR-9a (p=0.0214) and BAL TBdx (p=<0.0001) in QFN positive participants whereas in QFN negative this was only upregulated by BAL TBdx (p=0.0014). CD38 expression within $CD3^+CD5^{hi}$ cell population showed significant downregulation by BiP stimulation (p=0.0315) in QFN negative; in addition, this was further downregulated by BiP plus TLR-9a in both QFN negative (p=0.0003) and QFN positive (p=0.0109). Expression of IL-5Rα within $CD3^+CD5^{hi}$ $CD38^+$ population was significantly upregulated by BiP stimulation (p=0.0130) within QFN negative groups; BiP plus TLR-9a within both QFN negative (p=0.0429) and QFN positive (p=0.0091) and BAL TBdx within both QFN negative (p=0.0003) and QFN positive (p=<0.0001). Co-expression with Fas-L was detected in lower frequencies within $CD3^+CD5^{hi}CD38^+$ but upregulated by stimulation with BAL TBdx (p=0.0315) in QFN positive group compared to unstimulated cells. PD-1 expression was upregulated within both participant groups by BAL TBdx stimulation (QFN negative p=0.0014 and QFN positive p=0.0004).



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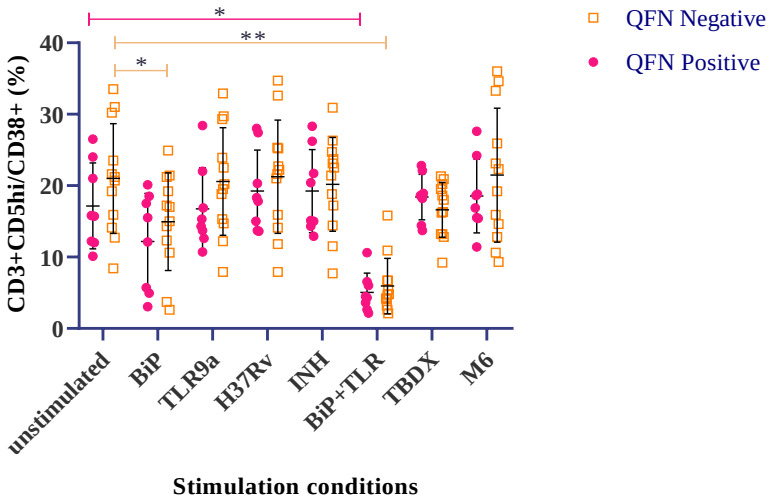

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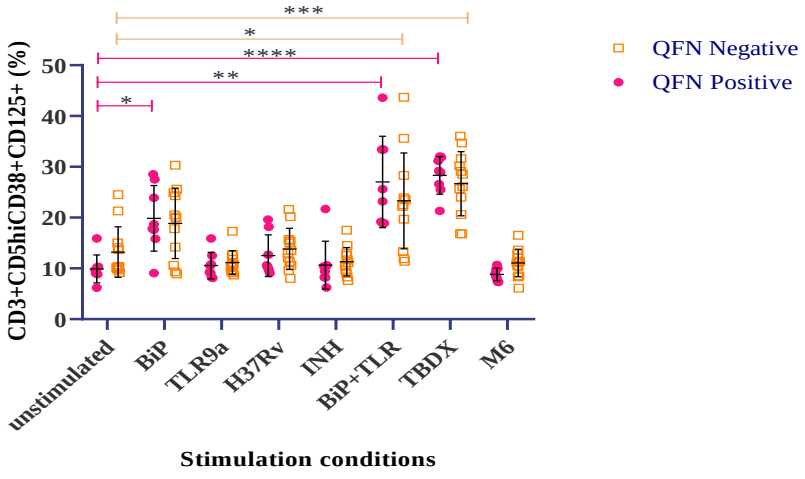

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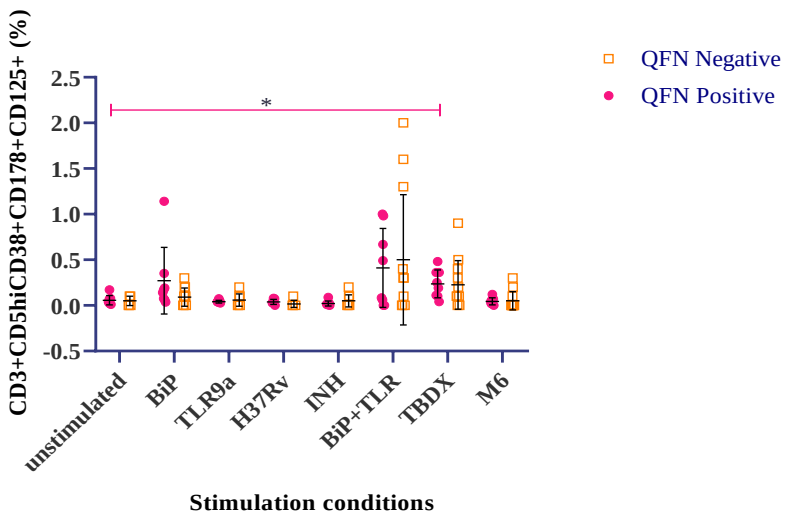

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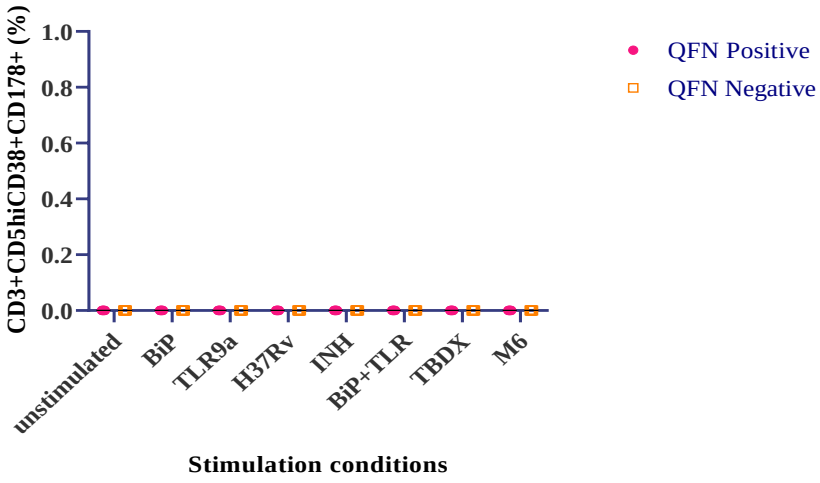

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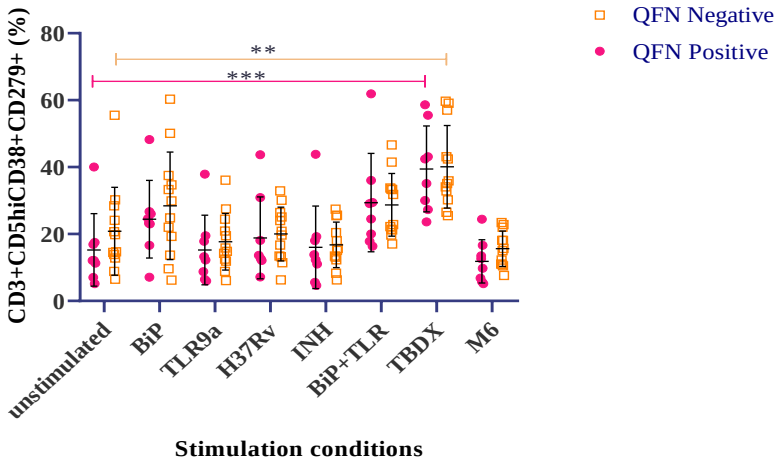


f)

Figure 4.5: Cell surface expression within T-cell subpopulations, where cell frequencies are represented as a) $CD3^{+}CD5^{hi}$, b) $CD3^{+}CD5^{hi}CD38^{+}$, c) $CD3^{+}CD5^{hi}CD38^{+}$, d)

$CD3^+CD5^{hi}CD38^+CD125^+CD178^+$, e) $CD3^+CD5^{hi}CD38^+CD178^+$ and f) $CD3^+CD5^{hi}CD38^+CD279^+$. Significant differences were tested through multiple *t*-test between participant groups and two-way ANOVA between unstimulated PBMC and other stimulation conditions within each group. Significant difference is indicated by asterisk where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001.

Cytokine profile in unstimulated and stimulated PBMCs

PBMC stimulation with different antigens induce both anti-inflammatory and pro-inflammatory responses

The heat map in Figure 4.6 shows relative secretion of cytokines from unstimulated and stimulated PBMCs. BiP stimulation induced strong responses of soluble Fas-L (sFas-L), IL-4, IL-5 and IL-13 whereas IL-10 response was reduced. Combination of BiP and TLR-9a increased secretion of IL-4, TGF-β1 and TGF-b3 in cultured PBMCs. Stimulation with H37Rv induced the strong response of pro-inflammatory cytokine TNF-α with a slight increase in IL-1β, IL-4, IL-5 and IL-13. This also increased anti-inflammatory mediators (Granzyme-B, sFas-L and IL-10). Stimulating PBMC with BAL TBdx showed the strong response of pro-inflammatory (IL-1β) and anti-inflammatory (IL-13) cytokines with reduced IL-10 and TGF-β2 responses. In contrast, pooled BAL (M6) reduced TGF-β2 responses, with no effect in the relative secretion of other cytokines. Furthermore, stimulating PBMC with TLR-9a reduced expression of pro-inflammatory TNF-α and anti-inflammatory sFas-L without affecting secretion of other cytokines. Further reduction in relative secretion of sFas-L and IL-13 is depicted during INH stimulation.

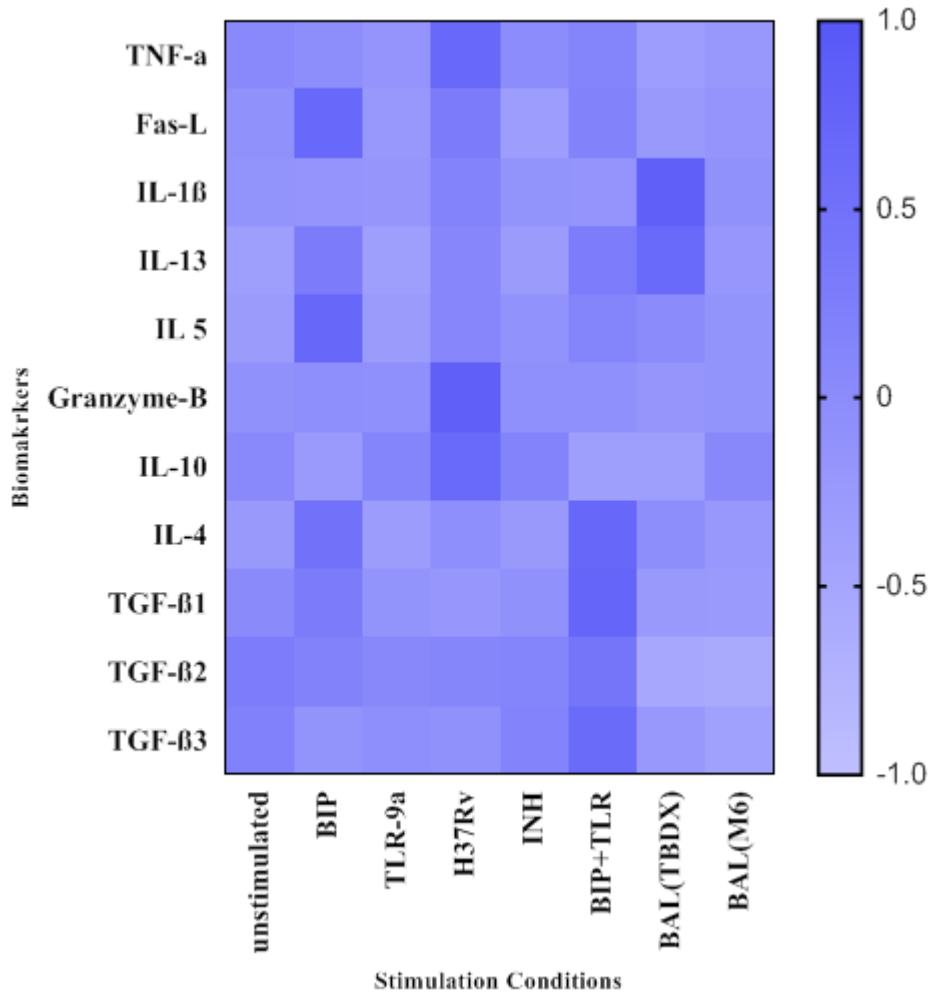
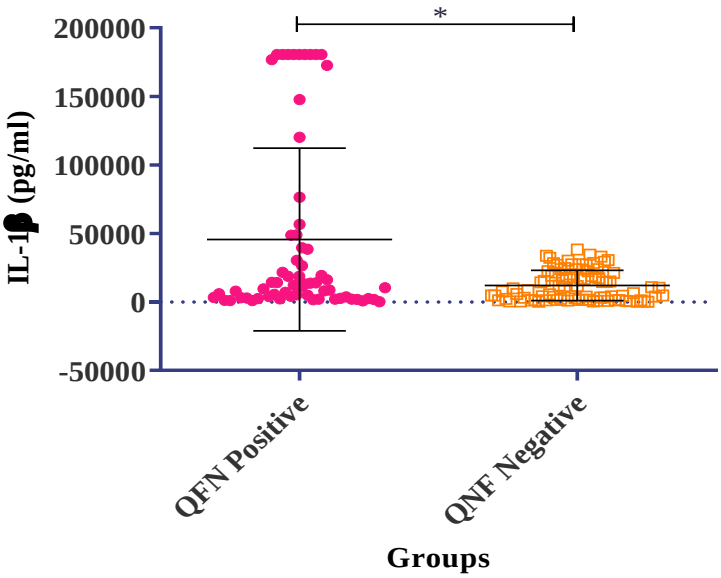


Figure 4.6: Heat map depicting relative secretion of cytokines from unstimulated and stimulated PBMCs. Stimulation condition mean values were normalised by row using feature scaling. Higher biomarker secretion is indicated by increased blue colour intensity.

Cytokine secretion profiles variation between QFN negative and QFN positive groups.

Comparison of 11 cytokine secretion between the two participant groups showed significant increase in IL-1β (p= 0.0162) secretion in QFN positive group compared to QFN negative group as shown in Figure 4.7. Secretion of IL-5 (p=0.0001), TGF-β2 (p=0.0135) and TGF-β3 (p<0.0001) was significantly upregulated by the QFN negative group.



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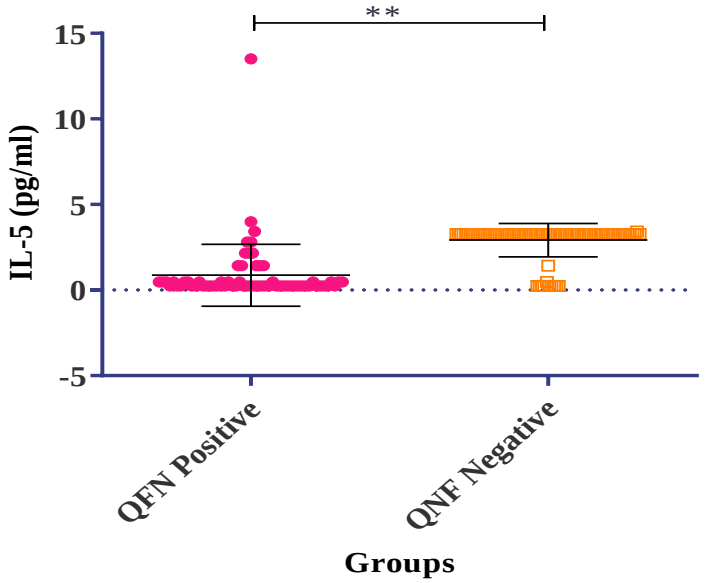

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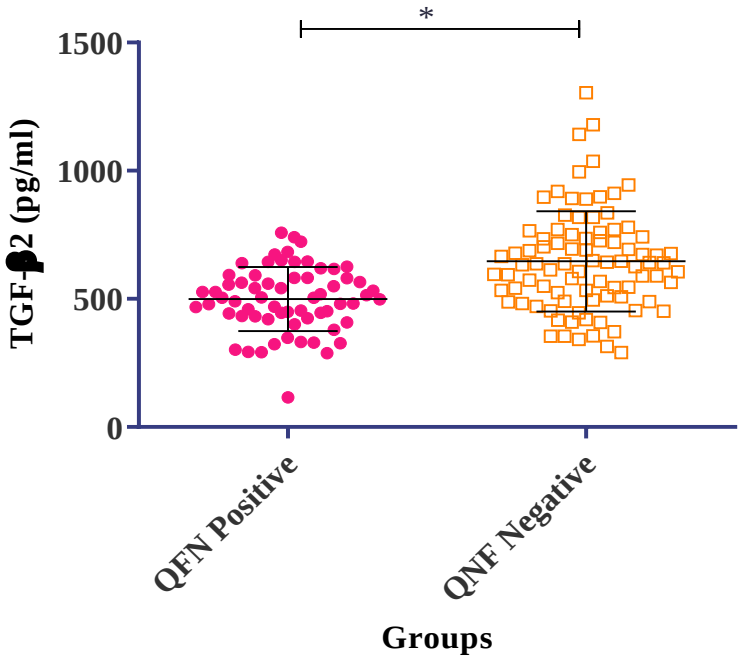

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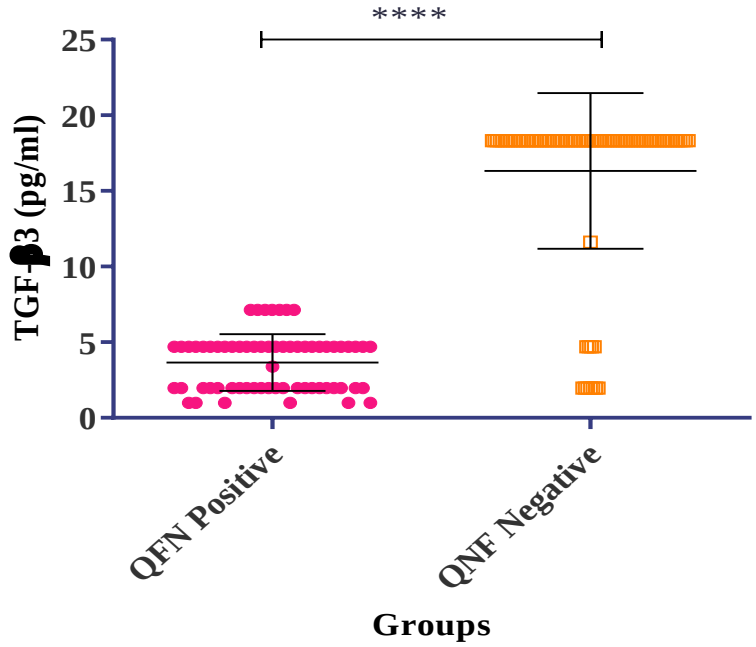
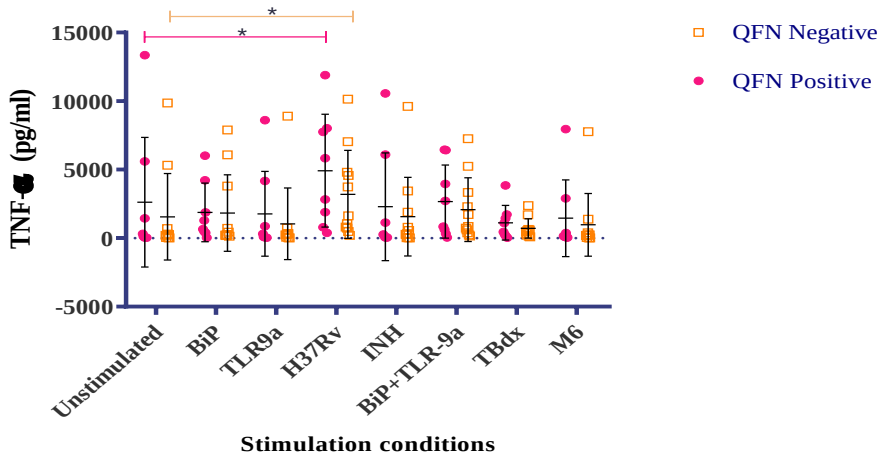


d)

Figure 4.7: Cytokine secretion profile between QFN negative and QFN positive participants a) shows IL-1β secretion, b) IL-5 secretion, c) TGF-β2 secretion and d) TGF-β3 secretion by stimulated PBMC. Significant difference is indicated by asterisk where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001.

Varying antigens elicit different cytokine profiles in PBMC

Figure 4.8 shows a significant difference in cytokine secretion elicited by various stimulation conditions within the two participant groups compared to their respective unstimulated cells. There was a significant upregulation of pro-inflammatory cytokine TNF-α in both QFN negative (p=0.0334) and positive (p=0.0114) participant groups following stimulation with H37Rv. Increased IL-1β by H37Rv stimulation in QFN positive (p=0.0001), additionally pooled BAL TBdx also induced significant upregulation of this cytokine in both QFN negative (p=0.0086) and QFN positive (p=0.0001) groups. Pro-inflammatory response IL-4 (QFN negative p=0.0006 and QFN positive p=0.0043) and IL-5 (QFN positive p=0.0002) was increased by BiP stimulation. In addition, stimulation with BiP plus TLR-9a further induced elevated levels of IL-4 in both participant groups (QFN negative p=<0.0001 and QFN positive p=0.0137). IL-13 showed high secretion following stimulation with BiP (QFN negative p=<0.0001 and QFN positive p=0.0006), BiP plus TLR-9a (QFN negative p=<0.0001 and QFN positive p=0.0022), BAL TBdx (both groups p=<0.0001) and H37Rv (QFN negative p=<0.0018 and QFN positive p=0.0006). Furthermore, stimulating with H37Rv upregulated secretion of IL-10 in both groups with a highly significant difference in QFN positive group (p=0.0004) compared to QFN negative (p=0.0385). Conversely, secretion of IL-10 was suppressed by BiP (p=0.0350), BiP plus TLR-9a (0.0120) and BAL TBdx (p=0.0179) in QFN positive group compared to unstimulated cells. In addition to H37Rv stimulation, Granzyme-B (p=0.0005) response was significantly increased in the QFN positive group. sFas-L was upregulated by BiP stimulation within QFN negative group (p=0.0402). Secretion of TGF-β variants showed differential response relative to other stimulatory antigens; TGF-β1 showed a significant increase in QFN negative group (p=0.0002) with BiP plus TLR-9a stimulation whereas this was not affected by other antigens. TGF-β2 showed significant downregulation by BAL TBdx and M6 stimulation with p=<0.0001 compared to unstimulated cells in both QFN positive and QFN negative groups. TGF-β3 was significantly suppressed by both BAL TBdx (p=0.0339) and M6 (p=0.0114) stimulation in QFN positive group.



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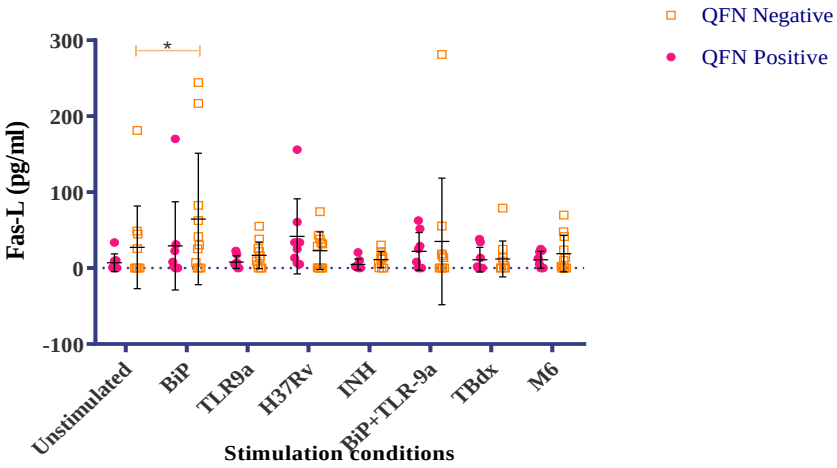

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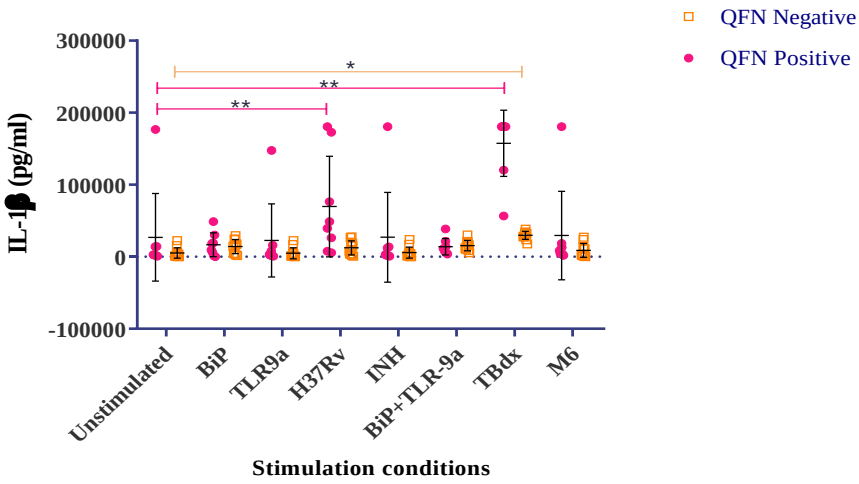

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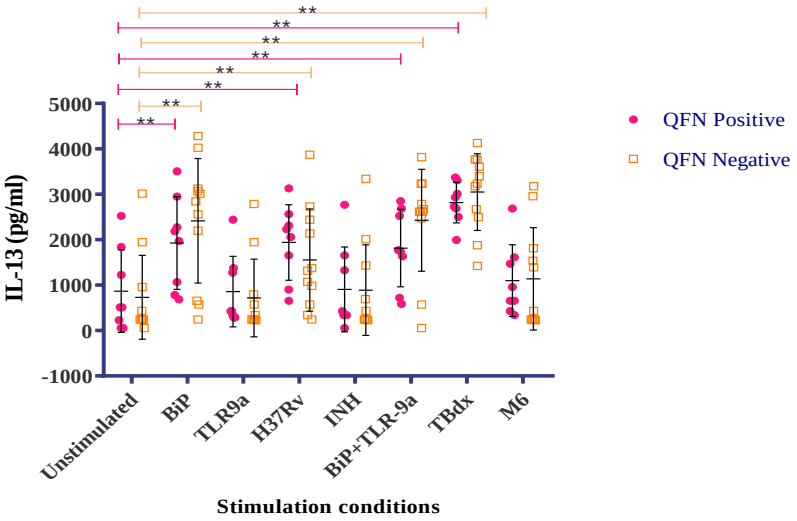

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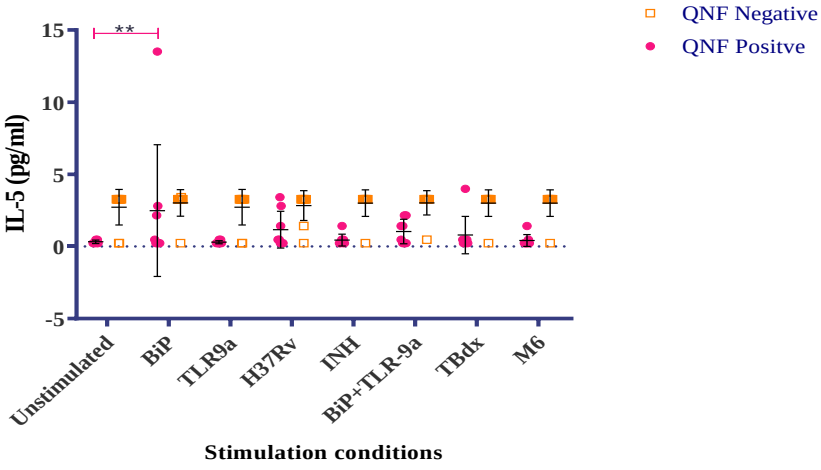

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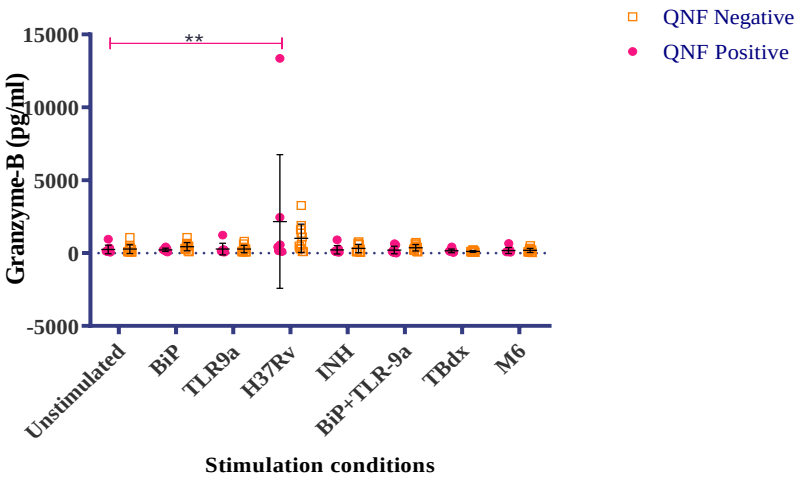

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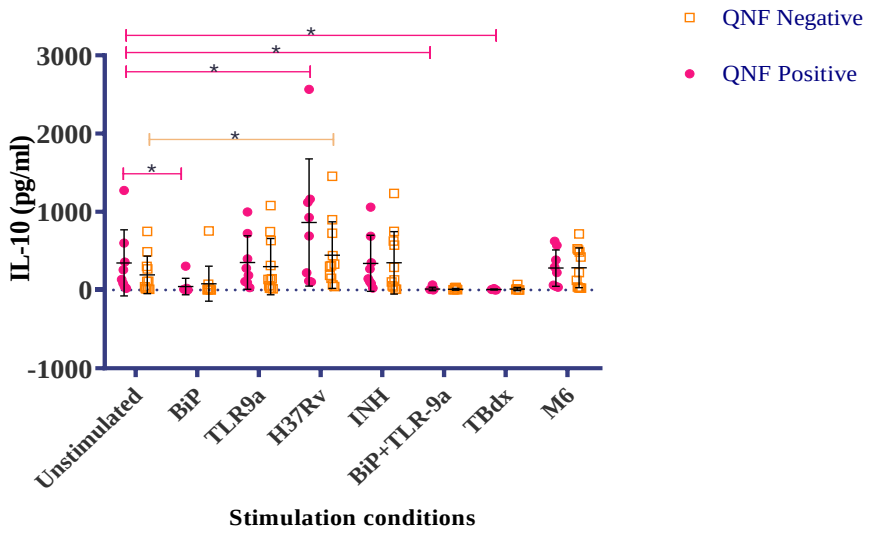

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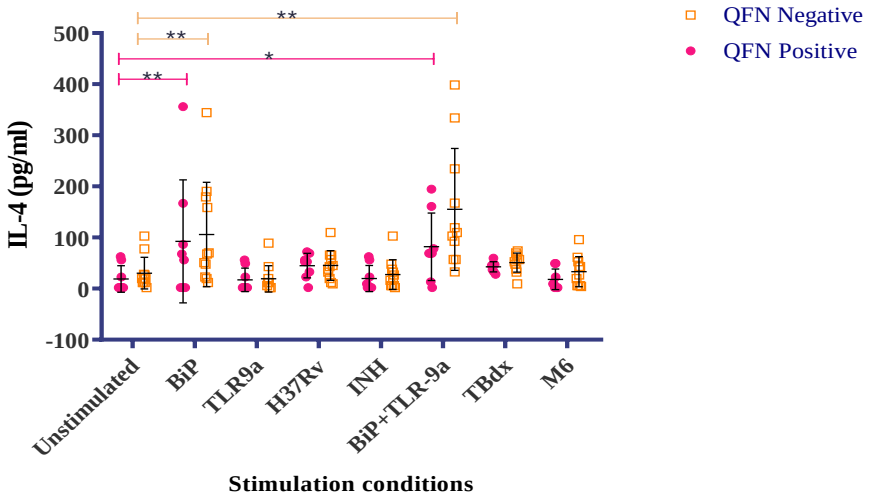

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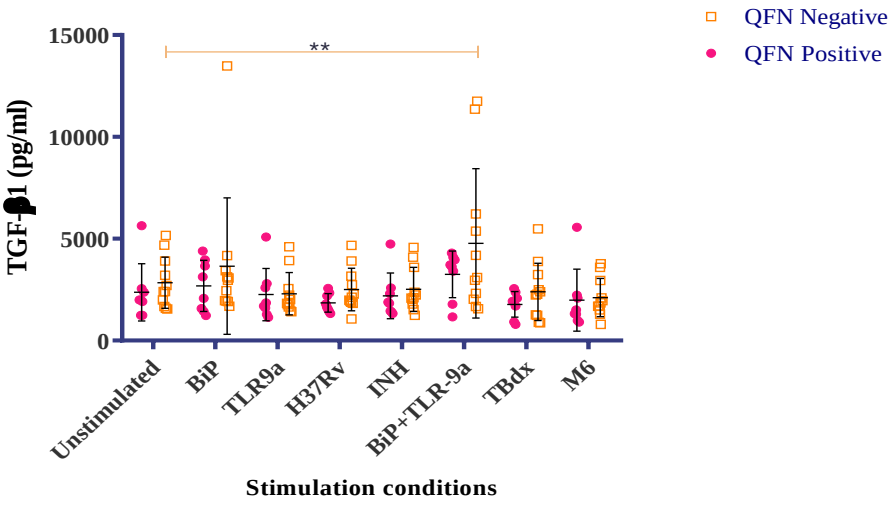

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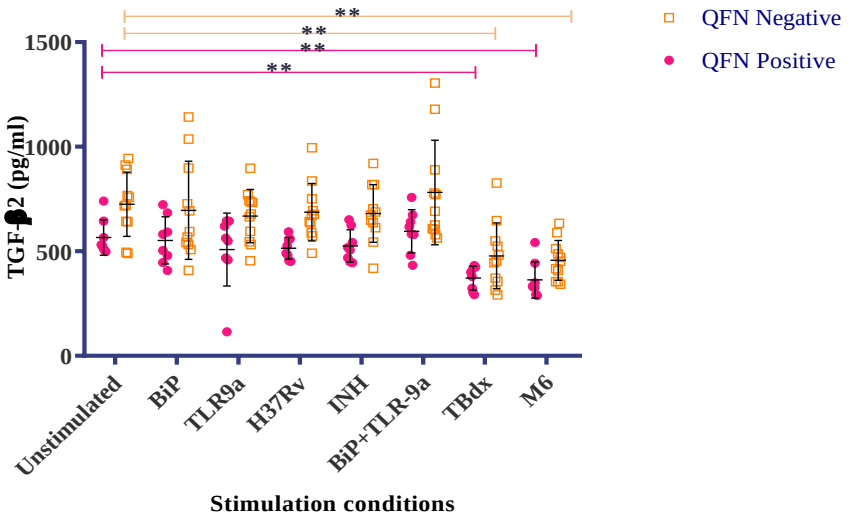

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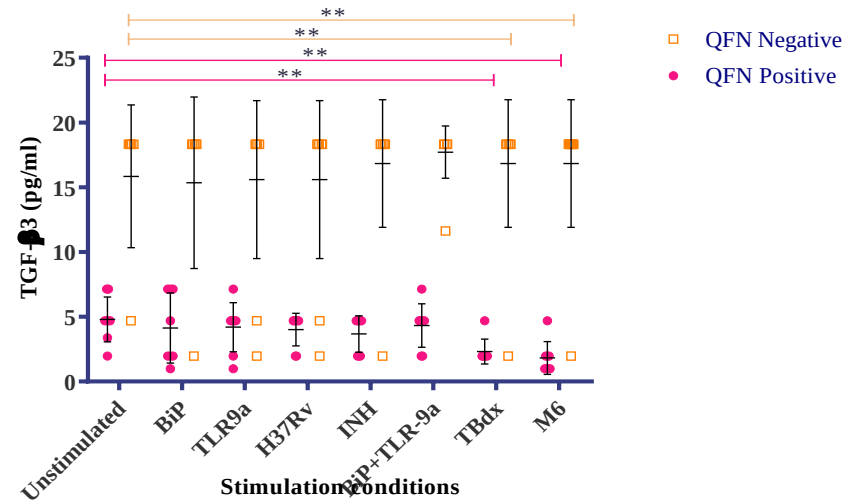


k)

Figure 4.8: Cytokine secretion profile during PBMC stimulation *in vitro* with different antigens; measured cytokines are represented by a) TNF-α, b) sFas-L, c) IL-1β, d) IL-13, e) IL-5, f) granzyme-B, g) IL-10, h) IL-4, i) TGF-β1, j) TGF-β2, k) TGF-β3. Error bars represent mean with standard deviation, significant difference is indicated asterisk where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001

Akt/ mTOR pathway regulation during cell stimulation with different antigens

Fluorescent intensity (FI) values were used to analyse the regulation of activated kinases. Table 4.2 highlights the statistical difference of activated kinases between QFN negative and positive.

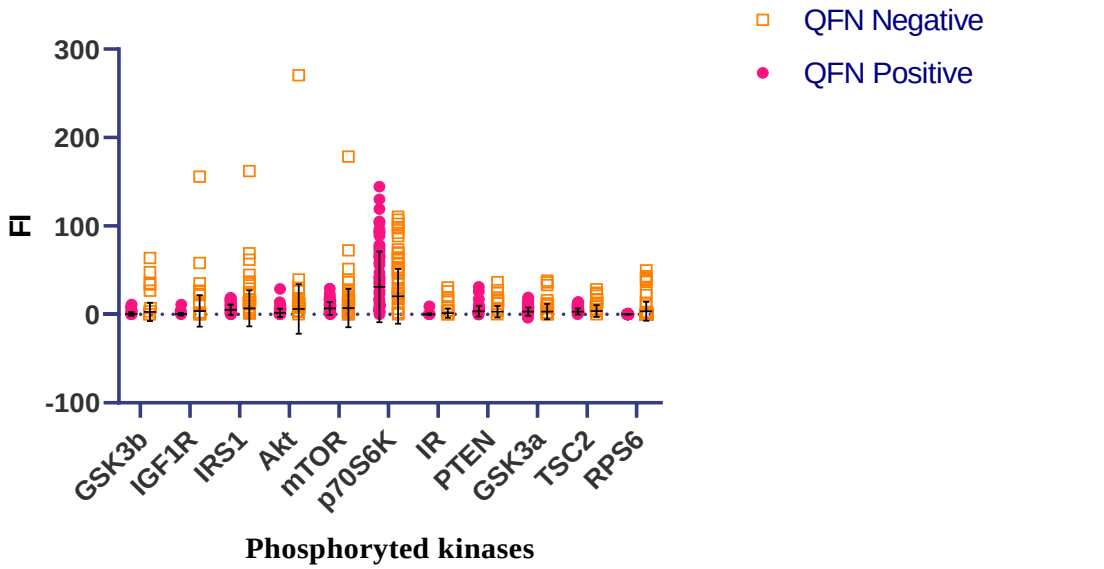
Table 4.2: Kinase activity between QFN negative and positive groups. Differences were calculated by multiple *t*-test analysis.

Kinases	Mean of QFN Negative	Mean of QFN positive	Adjusted p-value
GSK3b	2.8	0.6	0.5519
IGF1R	4.0	0.4	0.5608
IRS1	6.8	5.3	0.9810
Akt	6.2	1.8	0.7764
mTOR	7.3	6.7	0.9810
p70S6K	20.5	31.1	0.4425
IR	1.5	0.1	0.3749
PTEN	3.1	3.6	0.9810
GSK3a	3.1	3.0	0.9810
TSC2	3.9	3.3	0.9810
RPS6	3.4	0.0	0.1363

QFN=Quantiferon,

Figure 4.9 (a) summarises activation of kinases from QFN negative and QFN positive participant groups which showed no significant differences between the two groups. However, we found increased activation of ribosomal protein S6 kinase beta-1 (p70S6K) in both groups compared to other kinases. Mean Fluorescent Intensities (MFI) of phosphorylated kinases are highlighted in Figure 4.9(b) with HepG2 Insulin control as a reference standard of detection. Results showed increased activity of glycogen synthase kinase-3 beta (GSK3β), Insulin-like Growth Factor-1 Receptor (IGF1R), Insulin Receptor Substrate- 1 (IRS1), Insulin Receptor (IR), mammalian Target of Rapamycin (mTOR) and Protein Kinase-B (Akt) in QFN negative group. Activity of p70S6K was

highly increased in both groups. Kinases such as GSK3β, IGF1R and IR were not detected in QFN positive group while Ribosomal Protein S6 (RPS6) activity was undetected in both participant groups. There was no difference in mean activity of Phosphate and Tensin Homolog (PTEN), Glycogen Synthase Kinase-3 alpha (GSK3α) and Tuberous Sclerosis Protein-2 (TSC2) in both groups.



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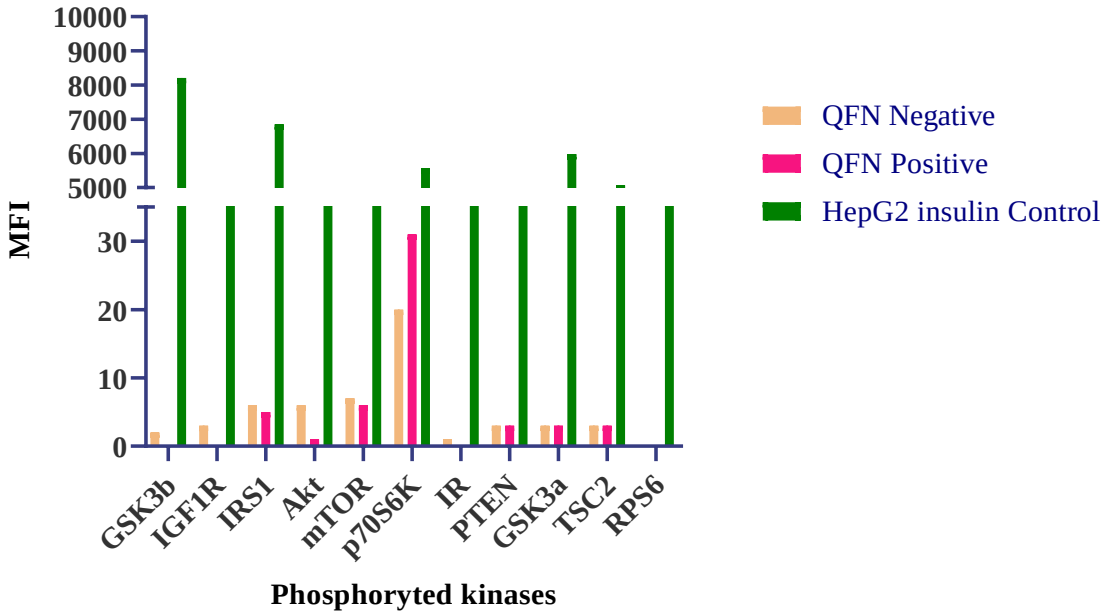
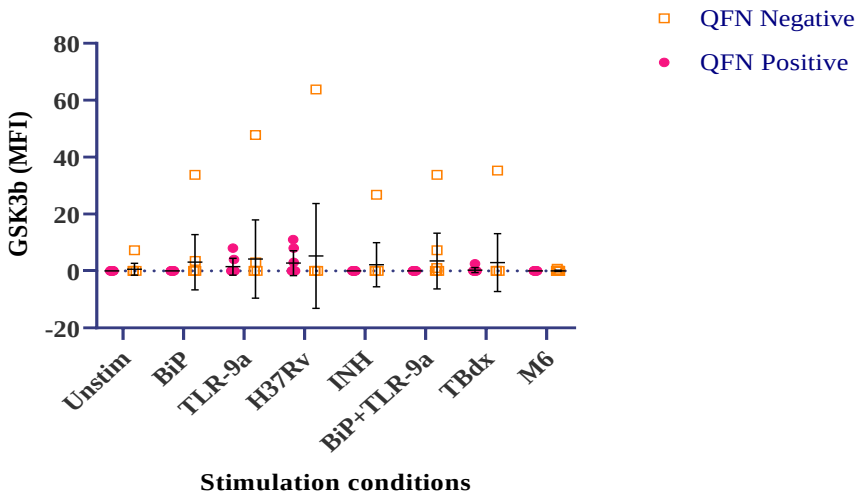


b)

Figure 4.9: Detection of phosphorylated kinases mediating Akt/mTOR pathway in PBMC isolated from QFN negative and QFN positive participants. Contingency graphs constructed using GraphPad prism 8 a) represents detected kinase FI between the two groups with mean and standard deviation

indicated by the error bars and b) represents MFI of phosphorylated kinases between the two groups with HepG2 insulin control as a reference standard.

Kinase activity of p70S6K was significantly increased by H37Rv stimulation in QFN positive group (p=0.0192) as shown in Figure 4.10. No further differences in kinase activity between the two participant groups were seen, except for increased activity of IRS1 (p=0.0179) and p70S6K (p=0.0446) in QFN positive with BAL M6 stimulation



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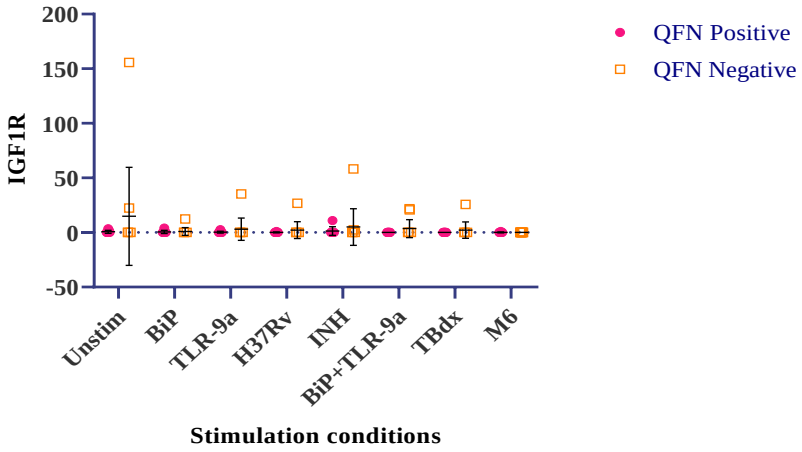

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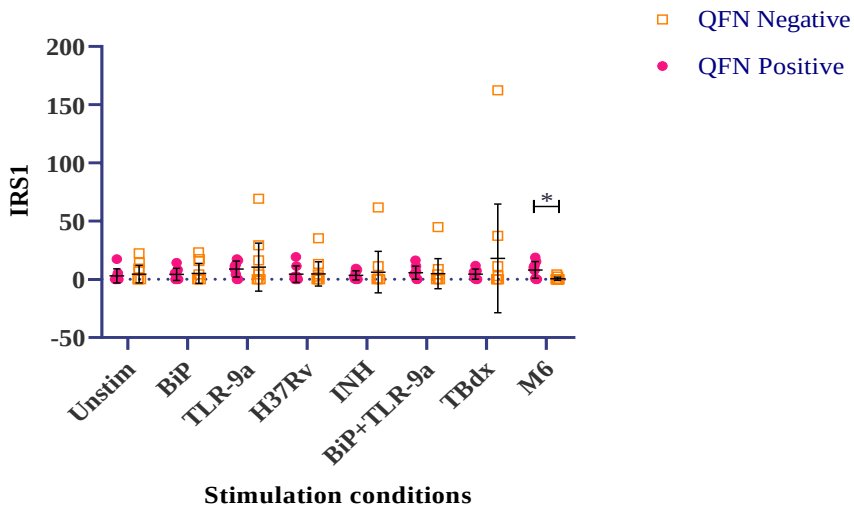

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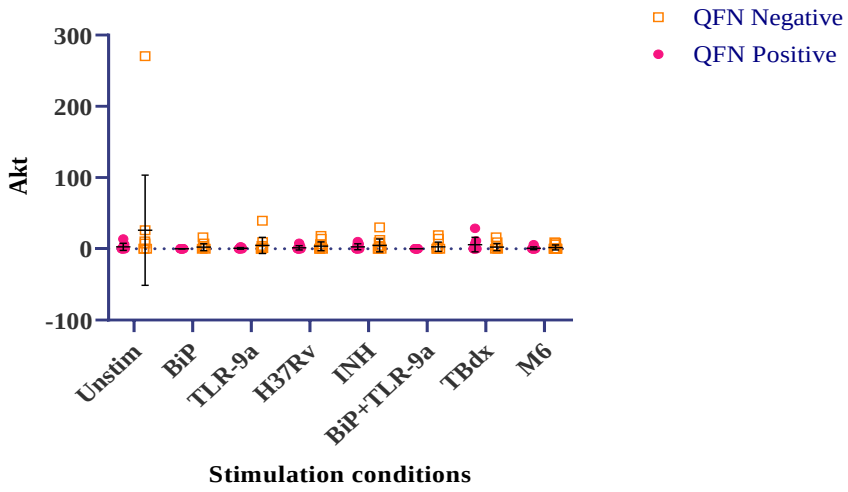

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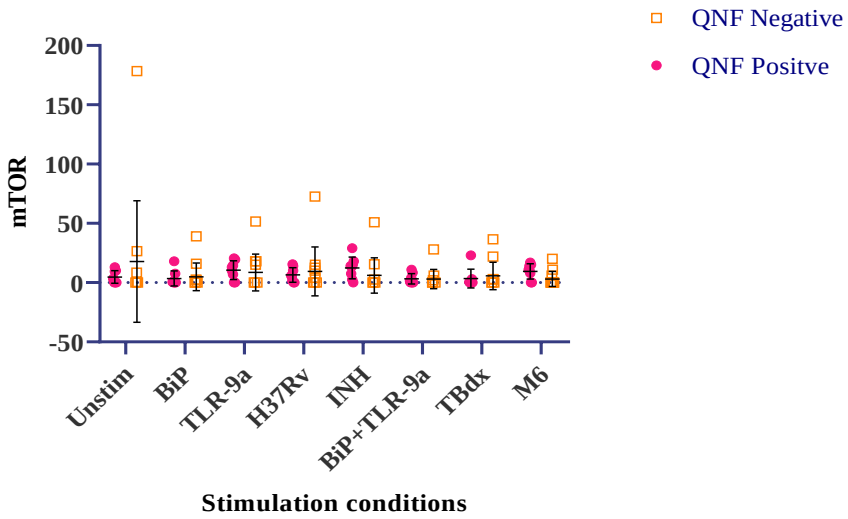

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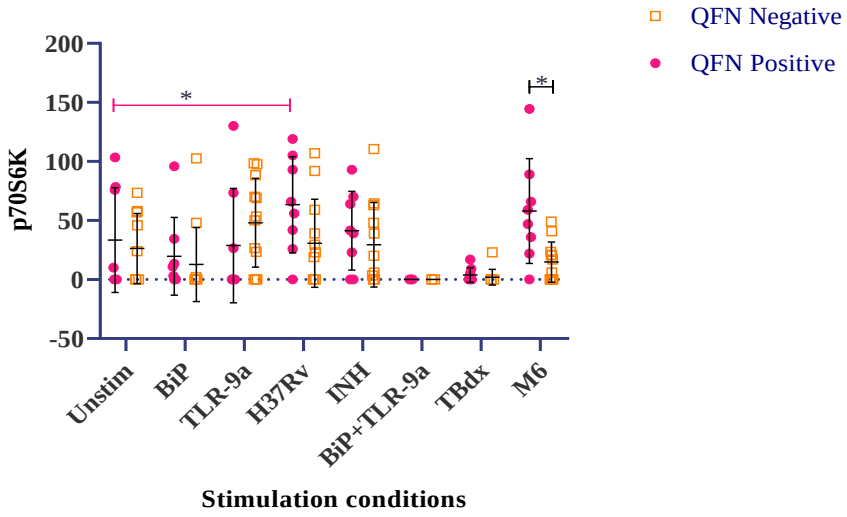

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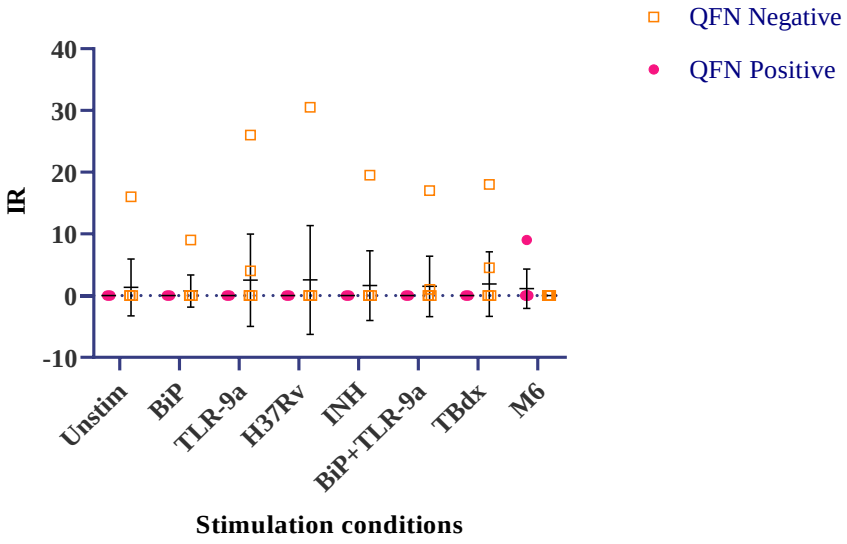

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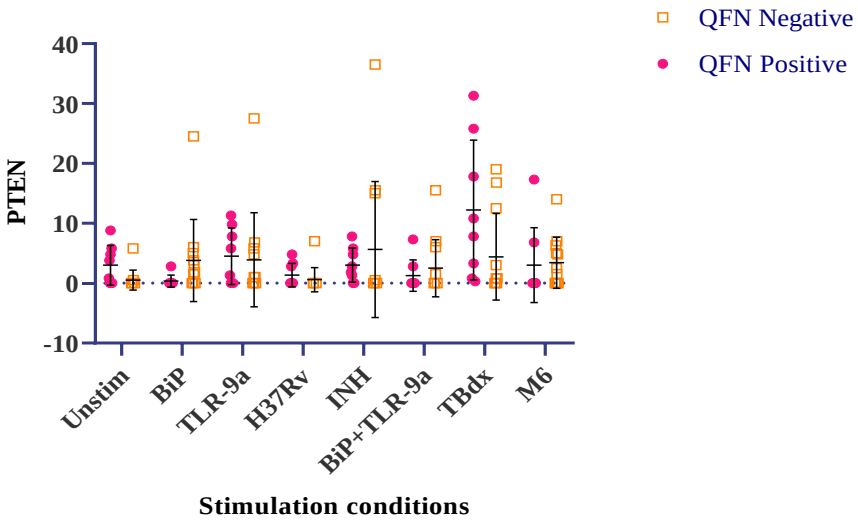

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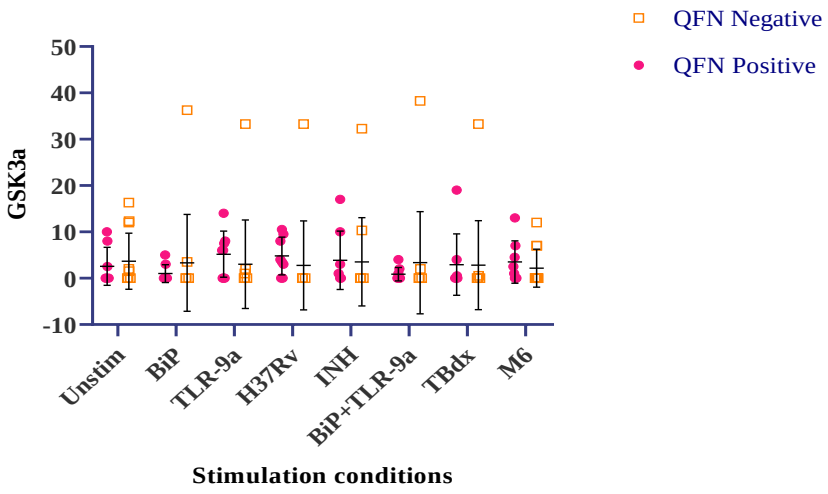

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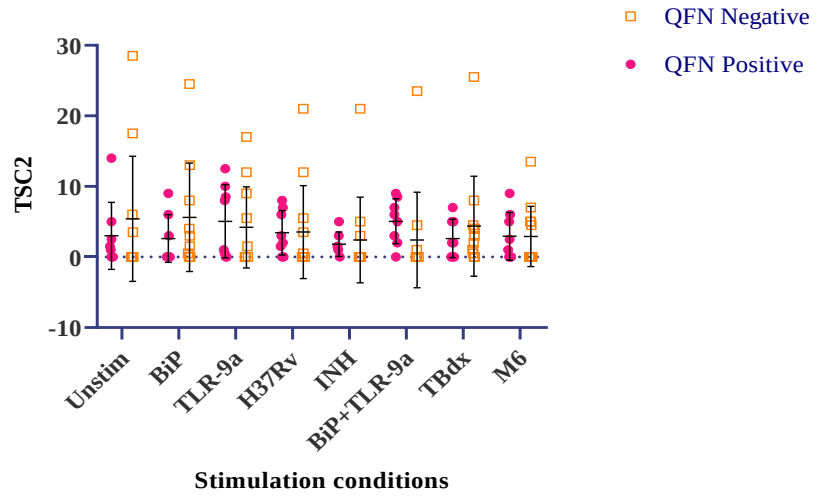

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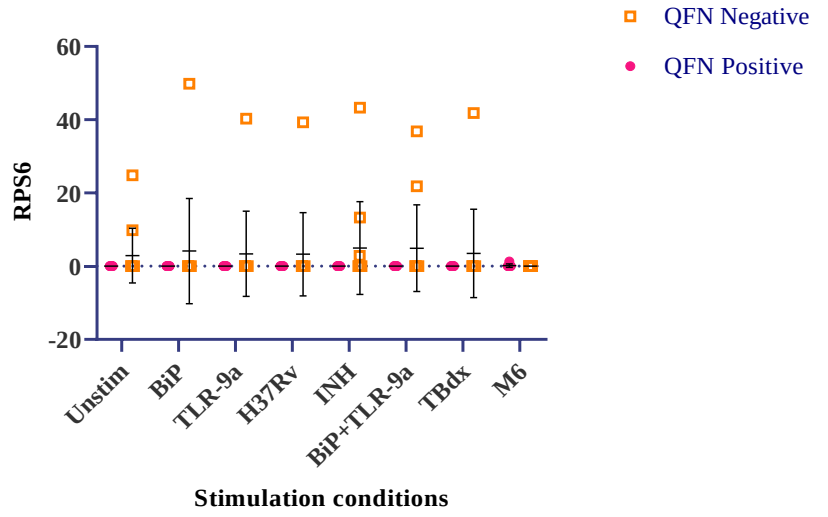

k)

Figure 4.10: Different stimulants elicit varying proportions of phosphorylated kinases involved in Akt/mTOR pathway as indicated by varying fluorescent intensities. Measured kinase activity is

represented by a) GSK3β, b) IGF1R, c) IRS1, d) Akt, e) mTOR, f) p70S6K, g) IR, h) PTEN, i) GSK3α, j) TSC2 and k) RPS6. Significant difference is indicated by the asterisk where * = p <0.05.

Discussion

The growing threat posed by tuberculosis disease on the world's population remains a challenge despite research advances made thus far. Progression to active TB disease is estimated at 5-10% of all latent TB cases (O'Garra et al., 2013). This highlights the need to further explore host mechanisms that may have potential in early eradication of this pathogen. This study focused on the effects of BiP to induce anti-inflammatory responses and upregulate expression of a killer/ regulatory phenotype on B-cells in comparison to other antigenic materials. Expression of death inducing ligands such as Fas-L on B-cells is an interesting concept which has been shown to be of significance during TB disease and other chronic inflammations (van Rensburg et al., 2017; Wang et al., 2017). These mediate apoptotic pathways of infected or metabolically exhausted cells and this form of innate immune response has been reported to aid in better control of *M.tb* infection (Nguyen and Russell, 2001).

Even though this killer phenotype on B-cells is not well described as it has been shown within different $CD19^{+}$ subpopulation such as $CD24^{+}CD27^{+}CD38^{+}$ and $CD5^{+}CD1d^{+}$. Here, expression of $CD19^{+}CD5^{+}$ on B-cells was assessed through flow cytometry; these cells remained unchanged by other stimulation conditions except in BiP stimulation (no statistically significant difference) and BiP plus TLR-9a (BiP+TLR-9a) stimulation (statistically significant difference) where their frequency was downregulated in both quantiferon negative and positive participant groups. This effect may be linked with secreted cytokine profile in these conditions, where cytokines such as IL-4, IL-5 and IL-13 which are known to directly affect immune cell proliferation were highly expressed. In particular; IL-4 and IL-5 cytokines are known to drive B-cell development towards immunoglobulin secretion (Pène et al., 1988). Furthermore, IL-5 was shown to induce Fas-L expression on B-cells within $CD5^{+}CD1d^{+}$ population, however, this effect was antagonised by IL-4 in these cells (Klinker et al.,

2013). The killer function mediated by Fas-L in B-cells is constantly associated with co-expression of IL5Rα which binds soluble IL-5 and mediate cell proliferation through transcription factor SOX-4 (Geijsen et al., 2001). Even though biological functions of this killer B-cell population during TB disease are not clearly defined; this study shows that co-expression of Fas-L and IL5Rα is inducible by recombinant BiP within $CD19^{+}CD5^{+}$ B-cell population in both *M.tb* unexposed and latently infected participants. According to Tang et al., (2016) BiP significantly upregulated expression of Fas-L and PD-L1 within $CD19^{+}$ cells in murin, however, in the present study, upregulation of Fas-L showed no significant difference with unstimulated cells. This discrepancy may be attributed to different species types and presence of other immune cells in our stimulations whereas Tang et al., (2016) studied BiP effect on isolated B-cells. This antigen showed synergistic properties with TLR-9a which binds TLR-9, a receptor that is known to be expressed by B-cells and plasmocytoid dendritic cells (pDC) (represent <0.2% of total PBMC) (Nair et al., 2012). In our cell stimulations, $CD19^{+}CD5^{+}CD38^{+}IgM^{+}L\text{-}5R\alpha^{+}$ cell frequency showed reduced frequencies for BiP and BiP+TLR-9a stimulations, suggesting that co-expression with Fas-L develops through upregulation of Fas-L within this B-cell population rather than upregulation of IL-5Rα from $CD19^{+}CD5^{+}CD38^{+}Fas\text{-}L^{+}$ as this population was not affected by most stimulation conditions. A study by Liu et al., (2018) showed the presence of *M.tb* in BAL and interestingly, our BAL fluid at TB disease diagnosis (TBdx) led to higher activation of $CD19^{+}CD5^{+}$ B-cells in PBMCs as indicated by $CD38^{+}$ expression. Additionally, upregulation of both $CD19^{+}CD5^{+}CD38^{+}IL\text{-}5R\alpha^{+}$ and $CD19^{+}CD5^{+}CD38^{+}IL\text{-}5R\alpha^{+}Fas\text{-}L^{+}$ B-cell populations by this stimulation indicated a changed environment mediated by *M.tb* infection since this effect was also observed with H37Rv stimulation, however, without affecting the frequency of $CD19^{+}CD5^{+}CD38^{+}IL\text{-}5Ra^{+}Fas\text{-}L^{+}$. In contrast, BAL fluid at M6 of TB treatment had no effect on expression of any marker within $CD19^{+}CD5^{+}$ B-cell population. This may be linked with a sterilized environment in the alveolar space with less inflammatory proteins and clearance of bacterial burden.

Balanced immune responses represent an important factor in disease elimination and prevention of autoimmunity. Our results are in accordance with previous studies suggesting that Mycobacterium

species (H37Rv) infection elicit both pro- and anti-inflammatory responses, particularly TNF-α and IL-10 respectively (Sahiratmadja et al., 2007). In the present study, TNF-α response was upregulated in both participant groups suggesting that early infection is highly mediated by this pro-inflammatory cytokine rather than IL-1β which were only upregulated in the quantiferon positive group. Upregulation of IL-1β response may be a function of memory response to *M.tb* antigens in this group. In both groups, H37Rv induced secretion of anti-inflammatory cytokines including IL-10 and IL-13 in both unexposed and quantiferon positive participants, however, in quantiferon positive group compensational anti-inflammatory response due to elevated IL-1β was mediated through secretion of an additional anti-inflammatory mediator granzyme-B which is known to induce apoptosis (Kataoka et al., 1998). Secretion of these mediators during *M.tb* exposure may provide a suitable niche for this pathogen to establish latent infection due to controlled pro-inflammatory response and lower frequencies of cells expressing apoptosis inducing ligand (Fas-L). However, expression of PD-1 was significantly upregulated by H37Rv within $CD19^{+}CD5^{+}$ B-cells in quantiferon positive group. This receptor induces programmed cell death in metabolically exhausted cells; this effect may provide insight and further understanding on factors involved in B-cell dysfunctionality which was shown by Joosten et al., (2016) during TB disease. Additionally, the frequency of B-cell subpopulation co-expressing Fas-L and IL-5Rα within $CD19^{+}CD38^{+}IgM^{+}$ was also shown to be diminished during TB disease treatment phase (van Rensburg et al., 2017). In this study BiP stimulation further enhanced immune responses mediated by T-helper-2 (Th2) cells through upregulation of IL-4 in both participant groups, however, IL-5 was only upregulated in quantiferon positive group. In addition to these pro-inflammatory responses induced by BiP, this also induced elevated levels of soluble Fas-L (sFas-L) in quantiferon negative groups and IL-13 in both quantiferon negative and positive group. sFas-L binds to Fas receptor expressed on both infected and metabolically exhausted cells to induce apoptotic pathways thus suppressing growth of *M.tb* (Oddo et al., 1998). In contrast, all TGF-β variants and granzyme-B secretion were not affected by BiP stimulation, however, TGF-β1 secretion was upregulated in a synergistic manner by BiP+TLR-9a stimulation.

T-cell mediated immune responses are known to be induced by secreted cytokines and antigen peptides presented on MHC complex by other cells (Medzhitov and Janeway, 1997). This study revealed differential T-cell modulation within both $CD3^{+}CD5^{lo}$ and $CD3^{+}CD5^{hi}$ in the presence of different antigens. Even though H37Rv did not result in any noticeable change within T-cell populations including both activation and expression of death inducing ligands, suggesting a delayed adaptive immune response driven by the increased IL-10 secretion (Fiorentino et al., 1991). However, activation of both T-cell subpopulations as indicated by $CD38^{+}$ expression was effectively downregulated by stimulating with BiP+TLR9a in both latently infected and unexposed participants. Suppressing T-cell activation during *M.tb* infection will maintain immune responses within the innate arm, additionally, with increased frequencies of cell populations expressing death inducing ligand by these antigens leads to increased control of *M.tb* infection through apoptosis (Hawn et al., 2013). Apoptotic pathways have been suggested to better control and limit spread of this pathogens within its host (Oddo et al., 1998; Srinivasan et al., 2014). In our study, the addition of BAL fluid TBdx to PBMCs affected T-cell profile both within $CD3^{+}CD5^{lo}$ and $CD3^{+}CD5^{hi}$; this reduced $CD3^{+}CD5^{hi}$ frequency in both participant groups while significantly upregulating the frequency of $CD3^{+}CD5^{lo}$ in QFN negative group. Previously, a study by Nambu et al., (2006) showed that elevated levels of IL-1β facilitate T-cell activation; this notion agrees with the observed levels of IL-1β secreted by QFN positive participants in this stimulation condition. BAL TBdx also upregulated expression of PD-1 and IL-5Rα on both T-cell subpopulations indicating metabolic exhaustion in these cells. Stimulating with TLR-9a did not affect the expression of any marker within both $CD3^{+}CD5^{lo}$ and $CD3^{+}CD5^{hi}$, this was mainly because these cells are known to lack the receptor that binds this antigen (Nair et al., 2012) and this was further shown by unchanged levels of T-cell cytokines IL-4 and IL-5 in this condition.

In the present study, we show that intracellular pathways are differentially regulated between quantiferon negative and positive participant depending on the type of antigen. The upregulation of p70S6K in QFN positive participants by H37Rv stimulation indicates activated mTOR pathway

which was shown to play a role in innate immune responses in a study by Schmitz et al., (2008). mTOR pathway is known to modulate different cellular mechanisms such as transcription, translation, mitochondrial metabolisms, cell-cycle progression and autophagy (Corradetti and Guan, 2006; Schmitz et al., 2008). This pathway was further reported to suppress IL-1β secretion after TLR-4 engagement, however, our results showed differential regulation of this cytokine with a high secretion of this cytokine by latently infected participants and unchanged secretion levels in healthy participants.

Taken together these results may provide insight into the modulation of (BiP induced) intracellular pathways between healthy and *M.tb* infected participants in response to bacterial antigens. This study paves the way for our increased knowledge on mechanisms involved during induction of killer phenotype in B-cells and leads to better understanding of potential pathways to be targeted to control TB disease.

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Chapter 5

Summary and Concluding Remarks

In the present study we hypothesised that extracellular Binding Immunoglobulin Protein (BiP) induces anti-inflammatory responses and induce B-cells to co-express a killer phenotype, Fas-L and IL-5Rα in healthy and *M.tb* exposed (latent TB infected) individuals. To further understand dysfunctionality associated with B-cells during TB disease and TB treatment, we further assessed secretion of this immunoregulatory protein during TB treatment and in healthy controls. Here we showed that immune cells are subjected to metabolic changes during early TB treatment which activates Unfolded Protein Response (UPR) due to high protein demand to mitigate inflammatory responses. This, in turn, leads to secretion of intracellular components amongst which includes BiP, which is also known to cause functional changes in different immune cells. Data obtained here indicated that progressive TB treatment resolves inflammation in TB participants leading to decreased UPR response as indicated by the gradual decrease of extracellular BiP concentrations towards the end of the treatment period.

We further aimed to determine the effect of human recombinant BiP on the expression of Fas-L on human PBMCs from participants with different *M.tb* exposure. Isolated PBMCs were subjected to a broad range of antigens to assess functional responses in comparison to unstimulated cells. Results from this study indicated differential responses by PBMCs in both cytokine profile and cell surface receptor expression. It was observed that extracellular BiP can induce higher frequencies of Fas-L expressing B-cells within $CD19^{+}CD5^{+}$ population than our widely known antigens. In this study, we also learned that BiP can act in synergy with TLR-9a to increase secretion of IL-4. This synergy between BiP and TLR-9a further downregulate the frequency of IL-5Rα^{+} expressing $CD19^{+}CD5^{+}$ B-cells in both healthy and latently infected participants while upregulating the frequency of $CD3^{+}CD5^{+}$ IL-5Rα^{+} T-cells.

Furthermore, the cytokine profile of immune cells during exposure to different antigens was established. We found that H37Rv infection induces both pro- and anti-inflammatory responses, however, this infection highly favours secretion of IL-1β during latent TB and TNF-α in healthy participants. Even though BiP decreases secreted IL-10 in both healthy and latently infected participants, it induces anti-inflammatory responses through upregulation of soluble Fas-L (sFas-L) in healthy participants and induces IL-13 in both healthy and latently infected participants.

Secreted cytokines are known to be tightly regulated by activated intracellular pathways; healthy and latently infected participants showed differential phosphorylation of kinases involved in both metabolic and transcription pathways. Stimulation with different antigens (including mycobacterial antigens) showed that secretion of pro- and anti-inflammatory cytokines are mediated by separate cellular pathways during H37Rv infection which are mediated through activation of p70S6K by mTOR pathway.

Combined, these results explore B-cell phenotype expression, cytokine profile and intracellular pathways in response to different antigenic stimulations and showed that extracellular BiP could be a target of choice to increase frequencies of Fas-L, IL-5Rα and PD1 expressing cells during active TB disease. This strategy may have greater impact as host directed therapy by early induction of apoptosis in infected cells and metabolically exhausted cells. Apoptotic pathways are beneficial as they facilitate cell death from within the cell by limiting important metabolites thus killing the pathogen during the process and limiting its spread.

Study Limitations

- For the most part, our study was limited to healthy and latently infected participants – inclusion of participants with active TB disease would have been beneficial to understand the role of BiP during active disease and stress even though we set out to first understand how

BiP influences B-cell related immune responses (which are best studied in uncompromised immune systems).

- Stimulation activity – a limited number of cells was available to activated kinases under different stimulation conditions. Addition of more cells could have aided to identify expression of kinases from both *M.tb* exposed and healthy participants
- Unbiased cell surface receptor analysis – we included limited B-cell surface markers to investigate the regulation of Fas-L within the B-cell population and excluded other regulator markers such as $CD24^{+}CD38^{+}$ and the expression of CD1d within our $CD19^{+}CD5^{+}$ B-cell population.

Future perspectives

- Future studies should include additional disease groups, like active TB cases and other lung diseases to pinpoint TB specific responses to BiP.
- Studies should also include additional markers of regulatory B-cells, i.e inclusion of anti-IL-10, the proposed hallmark of a killer/regulatory B-cells induced by BiP.
- Evaluation of the effect of BiP modulated immune responses to a multitude of *M.tb* strains in the context of antigen presentation and bacterial control.

Conclusion

This project highlighted the role of BiP in the induction of anti-inflammatory responses and a killer/ regulatory phenotype on B-cells during *M.tb* exposure. Our results represent a first step in establishing a platform for TB disease control, through limiting T-cell adaptive immune responses and possibly decreasing the rate of necrosis during infection. Establishing new targets directed at both intracellular pathway modulation and increased apoptosis factors may prove valuable as host directed therapy for improved *M.tb* infection control as these will decrease necrotic cell death and limit spread of this pathogen to infect more uninfected cells.

Appendix

A 1: Recombinant Human GRP78 (BiP) Protein.

Recombinant Human GRP78 (Bip) Protein

SPR-119A

Lot# 120914B

StressMarq Biosciences INC.
Discovery through partnership | Excellence through quality

Overview

Product Name	Recombinant Human GRP78 (Bip), native sequence Protein
Unit Size	50µg
Molecular Weight	≈78kD
Tested Applications	WB Control, ATPase activity, Binding assays, ELISA reference standard
Tag	No tag
Concentration	1mg/mL
Product Citations	PubMed ID: 23049684, 23448667, 23858031, 24853418

Technical Data

Purity	Affinity Purified
Expression System	E. coli
Certificate of Analysis	This product has been certified >90% pure using SDS-PAGE analysis and gamma globulin as the protein concentration standard. The protein has ATPase activity at the time of manufacture of 3.6µM phosphate liberated/30minutes/µg protein in a 100µL reaction at 37◦C in the presence of 20µL of 1mM ATP using a Malachite Green assay.
Storage Buffer	20mM Tris/HCl pH7.5, 0.45M NaCl, 10% glycerol, 0.5mM DTT
Storage Conditions	-20°C; 1 year+ Avoid freeze/ thaw cycle.
Shipping Temperature	Shipped on cold packs or ambient

Biological Description

Alternative Name(s)	BIP, Grp78, HspA5, MIF2, immunoglobulin heavy chain binding protein
Research Area(s)	Heat Shock, Chaperones, Organelle Markers, Trafficking
Sequence References	Gene ID: 3309; Accession Number: NM_005347; Swiss Prot: P11021
Cellular Localization	Endoplasmic reticulum lumen, Melanosome
Function	GRP78 is a ubiquitously expressed, 78-kDa glucose-regulated protein, and is commonly referred to as an immunoglobin chain binding protein (BiP). The BiP proteins are categorized as stress response proteins because they play an important role in the proper folding and assembly of nascent protein and in the scavenging of misfolded proteins in the endoplasmic reticulum lumen. Translation of BiP is directed by an internal ribosomal entry site (IRES) in the 5' non-translated region of the BiP mRNA. BiP IRES activity increases when cells are heat stressed (1). GRP78 is also critical for maintenance of cell homeostasis and the prevention of apoptosis (2). Luo et al. have provided findings that suggest GRP78 is essential for embryonic cell growth and pluripotent cell survival (3). In terms of diseases, GRP78 has been shown to be a reliable biomarker of hypoglycemia (Barnes), to serve a neuroprotective function in neurons exposed to glutamate and oxidative stress (4), and its protein levels are reduced in the brains of Alzheimer's patients (5). Also, the induction of the GRP78 protein that results in severe glucose and oxygen deprivation could possible lead to drug resistance to anti-tumor drugs (6, 7).

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Created/Modified: 13-Jun-2017

A 2: Toll-like receptor-9 agonist

Miltenyi Biotec

ODN 21798

Order no. 130-100-281
Order no. 130-100-280

Contents

1. Description

Components	**130-100-281:** 200 µg lyophilized ODN 1 mL 1× TE Buffer or **130-100-280:** 1 mg lyophilized ODN 1 mL 1× TE Buffer.
Description	P-class CpG oligodeoxyribonucleotide (ODN).
Product format	Lyophilized product without carrier protein or preservatives.
Sequence	dT*dC-dG*dT*dC-dG*dA*dC-dG*dA*dT* dC-dG*dG*dC*dG*dC-dG*dC*dG*dC*dC*dG * Phosphorothioate backbone
Endotoxin level	Low endotoxin (<1 EU/mg) as determined by kinetic Limulus Amebocyte Lysate (LAL) assay.
Storage	Store lyophilized product at –20 °C. Upon reconstitution, aliquots should be stored at –20 °C and are stable for 6 months. Avoid repeated freeze-thaw cycles. The expiration date is indicated on the vial label.

2. Background information

TLR9 is a prominent member of the toll-like-receptor (TLR) family recognizing pathogen-associated molecular patterns. TLR9 recognizes specifically unmethylated CpG motifs in bacterial DNA leading to activation of immune cells[1,2]. These effects can be mimicked by short synthetic ODNs containing unmethylated CpG motifs[3]. Several classes of CpG ODNs have been identified and can be distinguished by their effects on certain cell types[4]. A-class ODNs containing 5' and 3' G-rich stretches induce high levels of type I IFN but show low induction of B cell proliferation[5]. B-class ODNs activate B cells and TLR9-dependent NF-κB signaling in recombinant cell lines but show low induction of IFN-α. C-class ODNs induce high amounts of IFN-α and activate B cells[6]. The recently discovered P-Class ODNs show similar but superior properties to C-class ODNs.[7]

3. Applications

3.1 General applications

- CpG ODNs can be used for activation of immune cells, such as human PBMCs, murine splenocytes or isolated immune cells (e.g., B cells and pDCs).
- CpG ODNs can be used to activate signaling in TLR9-expressing recombinant cell lines.

3.2 Specific applications

- P-class ODNs activate both, B cells and pDCs with higher efficiency than C-class ODNs. P-class ODNs can form multimeric structures.

4. Instructions for use

4.1 Recommended concentrations

Recommended concentrations for use are

for human and murine immune cells: 0.05–2 µM

for recombinant cell lines: 0.05–10 µM

▲ An excessively high concentration of ODNs may result in decreased activity. Therefore, the optimal concentration range should be determined for individual assay systems.

4.2 Reconstitution protocol

1. Spin down pellet.
2. a) For 200 µg lyophilized ODN:
   To obtain a 200 µM solution resuspend pellet in 137 µL of 1× TE Buffer.
   ▲ Note: Alternatively, PBS or water can be used for reconstitution.

   b) For 1 mg lyophilized ODN:
   To obtain a 200 µM solution resuspend pellet in 685 µL of 1× TE Buffer.
   ▲ Note: Alternatively, PBS or water can be used for reconstitution.
3. Vortex and incubate overnight at 4 °C.
4. Store aliquots at –20 °C.

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A 3: BD anti-mouse Ig, κ negative control compensation beads

BD™ CompBeads

Technical Data Sheet

Anti-Mouse Ig, κ/Negative Control Compensation Particles Set

Product Information

Material Number:	**552843**
Component:	**51-90-9001229**
Description:	Anti-Mouse Ig, κ
Size:	6 mL (1 ea)
Storage Buffer:	Aqueous buffered solution containing BSA and ≤0.09% sodium azide.
Component:	**51-90-9001291**
Description:	Negative Control
Size:	6 mL (1 ea)
Storage Buffer:	Aqueous buffered solution containing BSA and ≤0.09% sodium azide.

Description

The BD™ CompBeads Set Anti-Mouse Ig, κ are polystyrene microparticles which are used to optimize fluorescence compensation settings for multicolor flow cytometric analyses. The set provides two populations of microparticles, the BD™ CompBeads Anti-Mouse Ig, κ particles, which bind any mouse κ light chain-bearing immunoglobulin, and the BD™ CompBeads Negative Control, which has no binding capacity. When mixed together with a fluorochrome-conjugated mouse antibody, the BD™ CompBeads provide distinct positive and negative (background fluorescence) stained populations which can be used to set compensation levels manually or using instrument set-up software. Since the compensation adjustments are made using the same fluorochrome-labeled antibody to be used in the experiment, this method allows the investigator to more accurately establish compensation corrections for spectral overlap for any combination of fluorochrome-labeled antibodies (without having to use valuable tissue samples or hard-dyed beads with potentially mismatched fluorescence spectra). Use of the BD™ CompBeads is highly recommended for use in all experiments using tandem dye (i.e., PE-Cy™7, APC-Cy™7, etc.) conjugates, which may have distinct spectral characteristics for each conjugate.

Preparation and Storage

Store undiluted at 4°C and protected from prolonged exposure to light. Do not freeze.

Application Notes

Application

Flow cytometry	Routinely Tested

Recommended Assay Procedure:

Note: BD Horizon™ V500 and AmCyan conjugated reagents can show significant differences in emission spectrum on stained cells and when captured on BD™ CompBeads. Thus, spillover values for these dyes evaluated with BD™ CompBeads may not provide correct compensation for cells. Therefore, single stained cellular controls are recommended to set up compensation for AmCyan and BD Horizon™ V500 reagents. BD Horizon™ V500-C has been modified to minimize these spectral differences and BD™ CompBeads may be used to determine spillover values for RUO antibodies conjugated to BD Horizon™ V500-C.

Without affecting compensation function, some lots may profile as a bi-modal histogram, which may be possible due to inherent light scatter and/or residual aggregation of the compensation particles. Optimization of instrument voltage or gating conditions may be helpful for improving histogram visualization.

This BD™ CompBeads Set has been tested with mouse Ig antibodies conjugated to various fluorochromes and analyzed using a BD FACS brand flow cytometer to ensure specificity and reactivity of the particles. See the specific instructions below on the use of the BD™ CompBeads Set:

1. Vortex BD™ CompBeads thoroughly before use.
2. Label a separate 12 x 75 mm sample tube for each flurochrome-conjugated mouse Ig, κ antibody to be used on a given experiment.
3. Add 100 µl of staining buffer [e.g., BD Pharmingen Stain (FBS), Cat. No. 554656 or BD Pharmingen Stain (BSA), Cat. No. 554657] to each tube.
4. Add 1 full drop (approximately 60 µl) of the BD™ CompBeads Negative Control and 1 drop of the BD™ CompBeads Anti-Mouse Ig, κ beads to each tube and vortex.
5. Add 20 µl of each prediluted antibody stock (diluted to a concentration optimal for staining 10^6 cells) to be tested on a given experiment to the appropriately-labeled tube. (Make sure the antibody is deposited to the bead mixture, then vortex.)
6. Incubate 15 - 30 minutes at room temperature. Protect from exposure to direct light.

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